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A STRAIN OF CONNECTIVE TISSUE SEVEN YEARS OLD.

By ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 51 TO 55.

(Received for publication, July 7, 1919.)

In July, 1914, Dr. Carrel reported the condition of a strain of connective tissue 28 months old, isolated from a fragment of heart extirpated from a chick embryo on January 17, 1912.[1] Today this strain is still alive. It has been under cultivation *in vitro* for a period of over 7 years and has undergone 1,390 passages.

The purpose of this article is to describe the technique employed in perpetuating the strain during the last 5 years and in measuring the increase of the tissue, the factors which influence the rate of growth, and the present condition of the strain.

Technique.

The technique does not differ fundamentally from the technique already reported.[2] The fragments of the old strain are allowed to grow undisturbed for 48 hours and are then divided into two parts and transferred to a fresh medium in the following manner.

The cover-glass is lifted with the heated point of a blunt cataract knife. It is then placed upon a piece of black glass. The fragment is extirpated by four clean cuts made with the blade of a sharp cataract knife within the area of new growth, and divided into two or three pieces as equal in size as possible. The pieces are transferred, with the point of the knife and a needle if necessary, to a bath of Ringer's solution. They are allowed to remain in the bath for about 45 seconds. Meanwhile the substances composing the medium are dropped upon a cover-glass and thoroughly mixed with the end of the cataract knife. Then the medium is spread over the surface of the glass, in

[1] Carrel, A., *J. Exp. Med.*, 1914, xx, 1.

[2] Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273.

order that the depth and area of the clot may be approximately uniform in all preparations.

The fragments of tissue are transferred by means of the knife point to the medium. They must be embedded thoroughly in it, without folding or curling. This step must be carried out rapidly to guard against embedding after coagulation has set in; that is, after 15 to 20 seconds, under ordinary conditions of room temperature and moisture. Coagulation is allowed to proceed and occurs in from 45 to 50 seconds. During this period, as well as during the period of washing in Ringer's solution, the preparations are kept under a large Petri dish in order to eliminate as nearly as possible chance bacterial contamination from the dust of the atmosphere.

After coagulation the cover-glass is inverted and placed on a hollow slide and held in place by a small quantity of vaseline. When coagulation does not occur promptly, the slide is prepared with vaseline in the manner described above, but instead of inverting the cover-glass over the slide, the slide is inverted and placed over the cover-glass, care being exercised not to touch the periphery of the medium. The slide is then picked up with its adherent cover-slip and set aside until coagulation occurs. This procedure prevents undue evaporation, which occurs if the medium is exposed for any length of time to the surrounding air, even though kept under the large Petri dish. The slides are sealed with paraffin melting at 56°C. and placed in the incubator at an average temperature of 39°C.

The medium used for perpetuating the strain is composed of equal volumes of chicken plasma and chick embryo extract. This combination produces a clot firm but not dense enough to interfere with the migration of the cells. Although a mixture of one volume of extract and two volumes of plasma constitutes a satisfactory medium, it has been observed that the new growth is less extensive. The clot is firmer and contracts more closely around the tissue fragment. It seems that the central portion of the tissue is not reached by fresh medium. The cells composing it die, and the necrotic tissue appears to retard the growth of the peripheral portion of the culture.

The plasma is obtained from adult chickens. The blood is taken from the carotid artery through an oiled glass cannula, received in chilled paraffined tubes, and centrifuged. Finally, the supernatant

plasma is pipetted off and kept in tightly corked paraffined tubes in cold storage at 4°C. The best plasma is obtained from young, healthy adult chickens, not over 2 years old, which have not been fed for a period of 24 hours previous to bleeding. Blood withdrawn from more recently fed chickens gives a turbid plasma rich in fat globules. Such plasma gives a hazy clot in which the cells do not develop so well as in a clear plasma.

The tissue extract employed is obtained from 7 to 8 day chick embryos. The embryos are first washed in Ringer's solution to remove the amniotic fluid and traces of blood which adhere to them after removal from the shell. Then the solution is drawn off in order to obtain an undiluted tissue juice. The embryos are minced in a watch-glass with sharp, curved scissors. The pulp is centrifuged for 10 minutes and the supernatant fluid obtained is drawn off.

The area of new growth is measured by means of a projection apparatus. In adjusting the condenser it is essential to guard against the action of the light rays upon the tissues, since the heat developed by concentration of the radiations is injurious. Diffused light sufficiently powerful to give proper illumination without any noticeable ill effect must be used. The image of the fragment of tissue is cast upon a sheet of paper, and by means of a pencil the outline of the original fragment and that of the new tissue can be traced. Shortly after the cultures are prepared, a drawing of the outline of the piece of embedded tissue is made, which requires not more than 20 seconds. The culture can then be returned to the incubator. Subsequent tracings, after growth is well established, are made at intervals on the same sheet of paper. They must be made rapidly because, after a short time, small droplets form and settle in the concavity of the slide. These droplets, usually after a period of from $1\frac{1}{2}$ to 2 minutes, coalesce and then no longer interfere with focusing. Such a long exposure of the tissue to the light has probably a retarding effect on the growth and should be avoided.

The rate of growth is expressed in function of the initial area for a given time interval. That is, the area of the newly grown tissue is equal to n times the initial area, as, for instance, it may be said that it has doubled or tripled, or that it is 10 times larger, within the same interval of time. Then, the rates of growth of two fragments of tissue

of unequal size can easily be compared. For example, the area of a 7 sq. cm. fragment, and of a 5.8 sq. cm. fragment, became in 48 hours, respectively, 99 sq. cm., and 81.5 sq. cm. The first one increased 92 sq. cm., the second only 75.7 sq. cm. However, the relation of the size of the growth to the size of the initial area is identical:

$$\text{No. 1, } \frac{99-7}{7} = 13.1 \qquad \text{No. 2, } \frac{81.5-5.8}{5.8} = 13.1$$

Each fragment has become 13.1 times as large as it was. The relation existing between the rates of growth of two fragments can be expressed in the form of a ratio, which is equal to $\frac{13.1}{13.1} = 1$ in the above example.

Factors Which Influence the Rate of Growth.

The strain is used chiefly for measuring the influence of different factors on the rate of growth of connective tissue. The value of the method depends entirely on the property of the parts of a divided fragment of tissue to grow at the same rate when they are cultivated in identical media. Therefore it is important to know what factors may cause two pieces of the same fragment to grow at unequal rates, and to recognize the presence of these factors after the fragments have been embedded in their medium.

As a rule, when two parts of a fragment of the strain which has been growing actively for 48 hours are cultivated in identical media, they grow at the same rate. However, it was found that parts equal or unequal in size coming from the same original fragment do not always grow at the same rate. The reasons for this difference have been determined in such a manner that examination of the pieces makes it possible almost always to foresee whether they will grow at the same rate, and then to discard the useless cultures.

Fragments identical in area may differ in thickness. This may be seen easily by microscopic examination, and sometimes by the naked eye. When a region whiter than the surrounding tissue is seen, it is probable that it is thicker and possibly necrotic. Microscopic examination of the tissue then shows a spot very much darker in color. Tissues presenting these characteristics should be discarded. They

probably would not grow at the same rate as tissues of homogeneous appearance, identical in area.

The condition of the edges of the fragments is also an important factor of the rate of growth. If the incision has been made through the growing area of the tissue, the periphery of the fragments has everywhere a similar appearance. The incision can be seen easily with the microscope if it has been made in places through the plasma not yet invaded by the new cells. Very often the old plasma is slightly folded. As the rate of growth is slower under these conditions such cultures should be discarded.

The value of the growth of a piece of tissue is compared only with the value of the growth of another piece of the same tissue. Therefore the absolute value of the growth is not of great importance, and the factors which do not modify the relative value of the growth can be neglected. However, these factors exist and may increase or decrease in a large measure the extent of the growth. In measurements made on 142 cultures, it was found that the surface of a fragment may increase from 4 to 40 times the initial size. These large differences in the amount of growth are due to the quality of the plasma and of the embryo extract used for the medium, to the previous condition of the strain, to the temperature of the incubator, and to many other factors. They are of no great interest, since they act at the same time on both the fragments which are to be compared. From a practical point of view, the factors which may influence independently the rate of growth of the two halves of an original fragment are almost completely eliminated, if the cultures containing pieces of old plasma or fragments of tissue of unequal size and thickness are discarded.

Present Condition of the Strain of Connective Tissue.

After more than 7 years of life outside the organism, the rate of growth of the strain of connective tissue is very active. This rate seems to have increased progressively during the 7 years (Figs. 1 and 2), but it is possible that it may be only an apparent increase due to modifications of the technique. During the 1st year the growth was slow and irregular, because it was not yet known that the presence in the culture medium of certain substances contained in embry-

onic juices is essential for the permanent life of tissues *in vitro*. As soon as the tissues were washed in Ringer's solution, or in salt solution every 2 days, and embryonic juice was used in the plasma of the medium, the rate of growth increased very much and became almost constant.[2] When the strain was 28 months old, the fragments of tissue which showed the maximum speed in growth increased in 48 hours 15 times their area. The 7 year strain increases more rapidly, as the area of a fragment may become in 48 hours 40 times larger than that of the original fragment. But this difference does not prove absolutely that the amount of new tissue produced in a given time is greater than it was 5 years ago. The medium used today is composed of one volume of plasma and one volume of embryonic juice, while 5 years ago it was composed of two volumes of plasma and one volume of embryonic juice. Since the medium is less dense, the growing tissue is probably thinner, and the increase in area does not mean the production of more tissue than that produced several years ago. The morphology of the cells has not varied. The photographs made 2 years ago and a few months ago show about the same number of mitotic figures (Figs. 3 to 5).

During the last 5 years the rate of growth fluctuated under the influence of the composition of the medium and the condition of the tissues. The plasma varied according to the age and the condition of the chicken and the embryonic juices were not of constant quality. Many other factors retarded or accelerated the growth of the tissue during periods of varying lengths of time. But as soon as the strain was again cultivated in normal medium, it grew at the normal rate. As has been shown previously, since the activity of the cells is a function of the medium in which they are living, it is readily modified by altering the composition of the medium. When the rate of multiplication of cells decreased and the amount of new tissue became small, the strain could be brought to normal growth within a few weeks. It was always found that the decrease in the rate of growth was due to deficient medium, or extract, or to the presence of alkali at the surface of the slides.

CONCLUSION.

1. A strain of connective tissue is still very active after more than 7 years of life *in vitro*.

2. The rate of growth of the fragments of tissue can be measured accurately and used for testing the action of many different factors on the growth of connective tissue cells.

3. The rate of growth of the strain is at least as rapid as it was 5 years ago, and may be more active.

4. The connective tissue cells appear to have the power of multiplying indefinitely in a culture medium, as do microorganisms.

EXPLANATION OF PLATES.

PLATE 51.

FIG. 1. Culture 9211–1. Passage 1080. Stained December 31, 1917. 48 hours growth. × 16.

PLATE 52.

FIG. 2. Culture 13310–1. Passage 1367. Stained May 23, 1919. 48 hours growth. × 16.

PLATE 53.

FIG. 3. Culture 5835. Passage 732. Stained April 16, 1917. 24 hours growth. × 275.

PLATE 54.

FIG. 4. Culture 9211–1. Passage 1080. Stained December 31, 1917. 48 hours growth. × 275.

PLATE 55.

FIG. 5. Culture 12784–1. Passage 1347. Stained April 25, 1919. 48 hours growth. × 240.

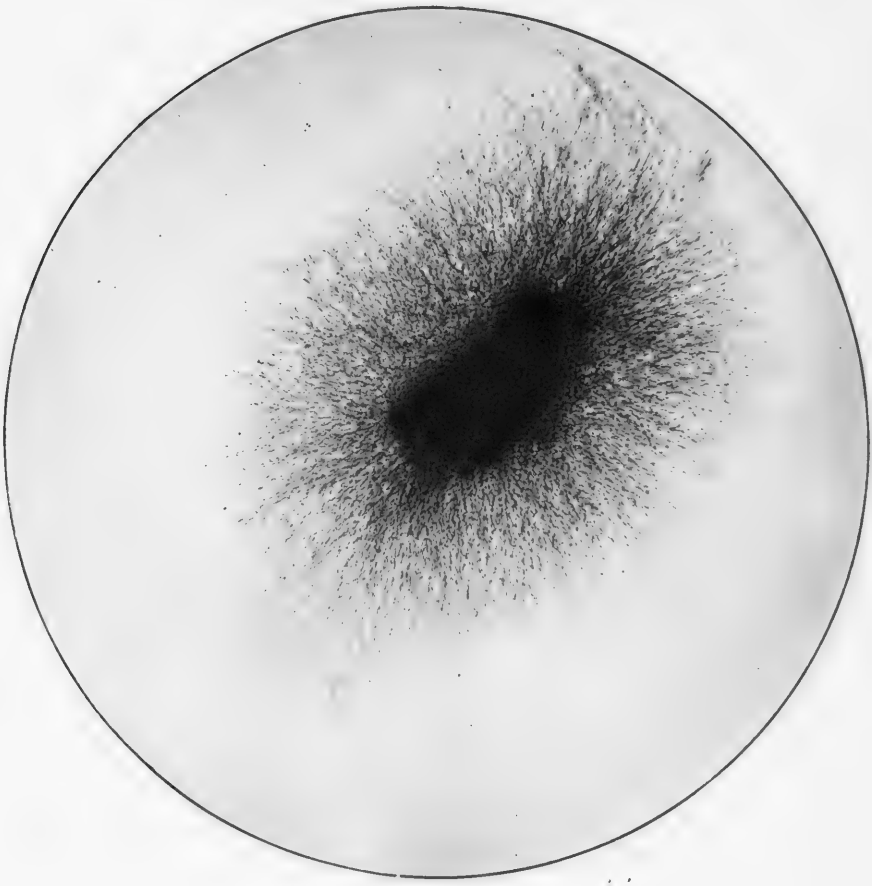

FIG. 1.

(Ebeling: Old strain of connective tissue.)

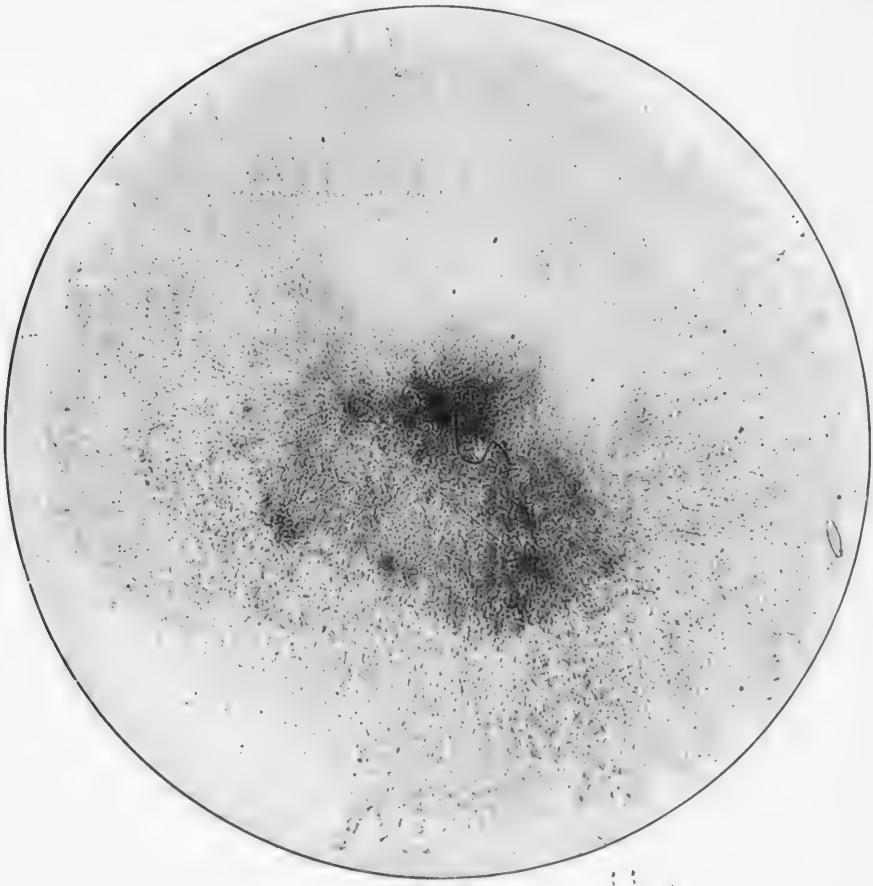

FIG. 2.

(Ebeling: Old strain of connective tissue.)

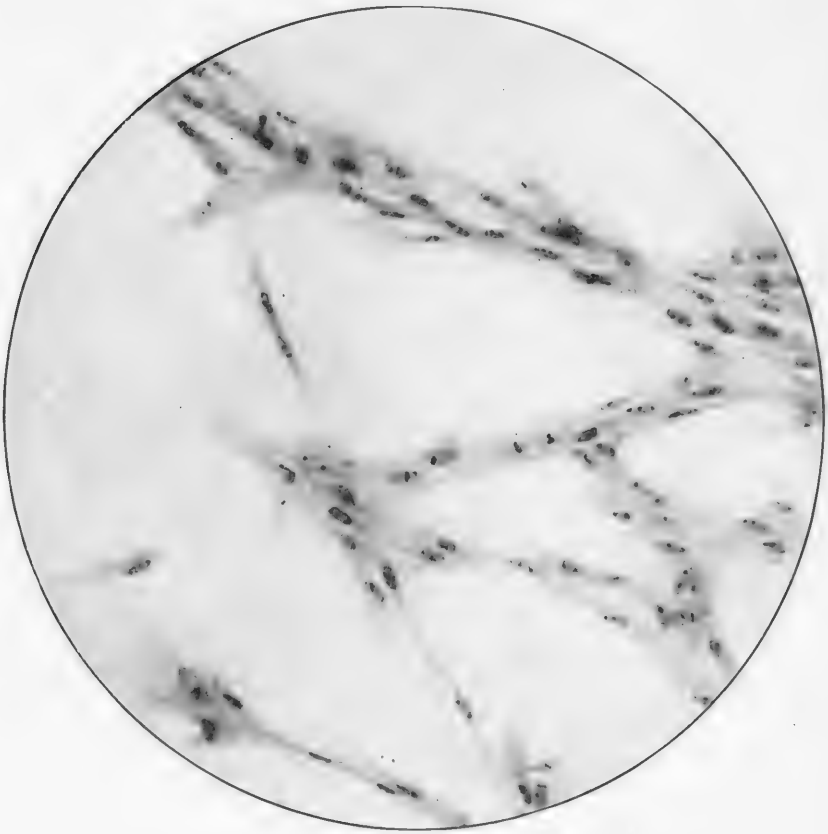

FIG. 3.

(Ebeling: Old strain of connective tissue.)

FIG. 4.

(Ebeling: Old strain of connective tissue.)

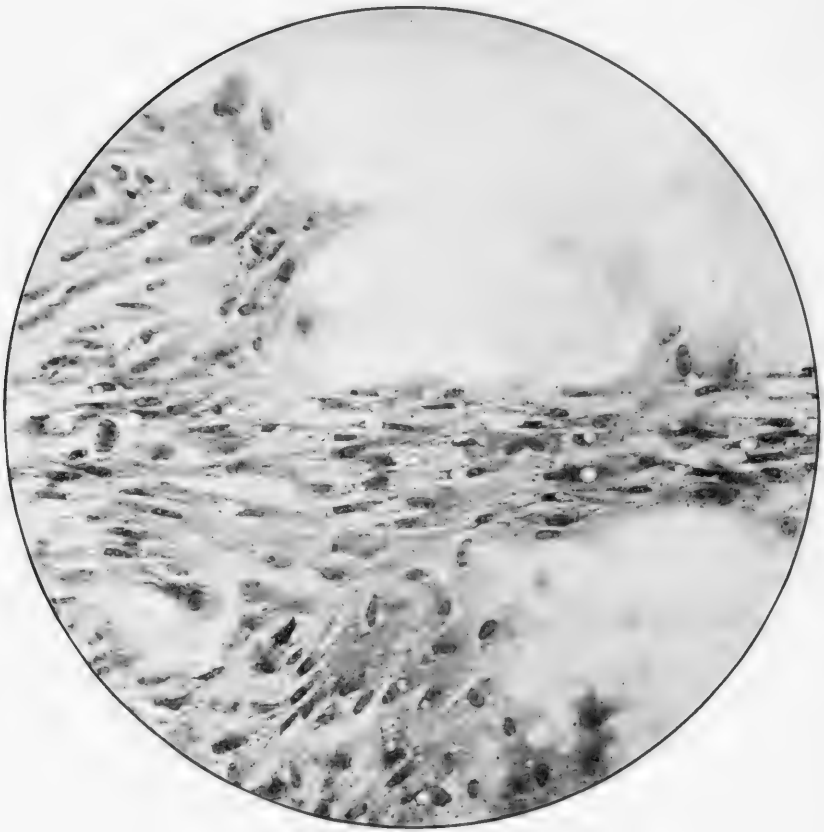

FIG. 5.

(Ebeling: Old strain of connective tissue.)

STREPTOLYSIN PRODUCTION IN CARBOHYDRATE MEDIA.*

By F. A. STEVENS, M.D.,

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(Received for publication, July 15, 1919.)

The inhibition of the hemolytic power of streptococci in media to which fermentable sugars have been added has been commented on by Ruediger (1), Sachs (2), Kuhn (3), Lyall (4), Davis (5), and Sekiguchi (6). After extended experiments Sachs concluded that in dextrose media the growth of the streptococcus was inhibited by the acid produced and the production of the hemolysin was correspondingly diminished. Kuhn, however, explained the effect of glucose by the theory that in carbohydrate media which were fermented by streptococci the metabolism of the organism changed so that the red cells were spared the direct lytic action of the bacteria. Although this in no way accounts for the diminished amount of hemolysin in filtrates of dextrose cultures, it was the first intimation that the protein-sparing action of carbohydrate might be applied to this phenomenon. Kendall and Farmer (7), studying the nitrogen metabolism, found that this principle was true in bacterial as well as animal physiology. Brown (8) has inferred that as there is undoubted evidence that streptolysin is a product of protein metabolism (it is thermolabile, can be filtered only through the coarsest filters, and is non-dialyzable), "in the presence of fermentable sugar the protein metabolism of the streptococci is reduced to a minimum and for this reason the formation of streptolysin is reduced or inhibited altogether." He suggested also that the acids formed might prevent the formation of streptolysin by acting on the growing streptococci, that the acids might inactivate the streptolysin during its formation, and, furthermore, might render the blood corpuscles insusceptible to hemolysis. It is possible on this basis to explain the action of sterile filtrates.

* From a report to the Surgeon General of the Army by a board of officers convened to study respiratory diseases.

In 1903 Schottmüller (9) noted that pneumococci and certain streptococci constantly produced green colonies on blood agar plates. Ruediger (1) attempted to show that this discoloration was due to acid production, and since the ferric chloride test on glucose broth cultures showed a reaction similar to that of lactic acid which he found would discolor blood media, he concluded that the green color was due to the action of lactic acid on red cells. He further states that not only did the addition of glucose increase the ability of pneumococci and streptococci to produce green colonies but that hemolytic cocci failed to hemolyze and after 36 to 48 hours incubation were surrounded by a distinct green halo. Cole (10) and Blake (11), working respectively with pneumococcus and *Streptococcus viridans*, discredited the action of acid in this reaction and showed that it was probably due to some inherent oxidative process acting on the cells immediately surrounding the colonies. Davis (5) found that after the addition of calcium carbonate to dextrose blood plates the average size of the zone of hemolysis was as large as on plain blood agar. By studying the solution of the carbonate particles and red cells simultaneously he concluded that the production of hemolysin and acid was coincident. Sekiguchi (6) stated that the hemolysin production was hindered by the glucose and not checked or destroyed by the acid. Finally, Brown (8) has observed that greenish or brownish discoloration similar to the alpha appearance is not produced by beta or gamma type of streptococcus in dextrose blood agar, but if there is sufficient fermentation the entire plate may be browned, first in the neighborhood of the colonies. The most noticeable effect was the inhibition of hemolysis by the actively hemolytic type. He further showed that acids produced in dextrose might account for some of the hemolysis of the gamma type streptococcus in poorly buffered media.

The reaction of the culture media for the production of streptolysin was described by Lyall (4) as optimum at 0.3 per cent acid to phenolphthalein, but he found inhibition if he varied the reaction much on either side of this point. M'Leod (12) obtained the greatest production in media distinctly alkaline to litmus. Since cultures become excessively acid in sugars which are fermented, calcium carbonate was added by Sachs (2) and Lyall (4), and the hemolysin determined. Lyall found that there was still marked inhibition. Sachs' tables show but little variation from the ordinary culture media except that the lysin was active slightly longer; by the addition of lactic acid to media before inoculation he shortened the life of the hemolysin by half. Braun (13) added acid and alkali to filtrates and after 6 hours incubation the alkaline preparation was most hemolytic; a filtrate obtained from a serum broth culture was made alkaline and incubated with a quantity of the original filtrate as a control. Both results were similar. Braun (13) and Sachs (2) concluded that the ability of the streptococcus to form acid was not to be identified with its hemolysin-producing power. That sufficient acid is formed in dextrose media to hemolyze red cells was shown by Sekiguchi (6); a 24 hour culture heated at 60°C. was not hemolytic after neutralization.

Methods.

The most important factor in the production of strong hemolysin is the media employed for the growth of the streptococcus. After testing various sera and broths M'Leod (12) considered beef infusion peptone broth with the addition of 20 per cent inactivated horse serum the most satisfactory, since he was able to obtain filtrates which were strongly hemolytic and fairly constant. In the following experiments a similar medium was used with 2 per cent peptone. No difficulty was experienced at any time in obtaining hemolysins and the filtrates were very active if obtained through new Mandler filters after 10 to 14 hours incubation. Since the object of the study was to observe the effect of dextrose and other fermentable sugars on the lysin production, it seemed necessary to measure the acid in the cultures at stated intervals. This was accomplished by means of a series of phosphate mixtures prepared according to Sörensen (14). To overcome the interference of the protein in the heavy serum media, 0.25 cc. was removed and diluted to 10 cc. before the indicators were added. The facts concerning the growth of the streptococcus were established on plate counts usually taken every 4 hours. It is realized that colony counts are open to much criticism, especially when an organism is counted which occurs in chain formation; films were made, a large number of groups of cocci was counted, and the number of individuals in each specimen was averaged. This factor usually varied from 4 to 7. In view of these facts, since the purpose was to obtain an idea of the relative growth of the organisms, the counts obtained by multiplying this number by the calculated colonies per cubic centimeter of the culture were sufficiently accurate. Hemolysis was determined by a series of tubes set up with increasing amounts of sterile filtrate or, as in the determination of hemolysin production, with the supernatant fluid obtained after centrifuging a portion of the culture at high speed for 10 minutes. 1 cc. of a 5 per cent suspension of washed human cells was added to each tube, and after an hour at 37°C. all were made up to 5 cc. volume and the undissolved cells were counted in a Levy chamber; the degree of red cell destruction was calculated in percentages by comparison with a standard control. Such counts are accurate since the occurrence

of shadow cells observed in hypotonic salt solution is rare; a perfect curve can be constructed from the degrees of hemolysis in the increasing amounts of hemolytic filtrate.

EXPERIMENTAL.

The effects of sugars were first observed on a series of plates made with 5 per cent defibrinated human blood and 1 per cent carbohydrate. The streptococci were obtained in pure culture from acute empyema fluids; five strains were chosen which gave a beta type hemolysis and had a final hydrogen ion concentration of pH 5.2 to 4.9 in dextrose broth. In liquid media they fermented dextrose, maltose, saccharose, lactose, and salicin, but did not so utilize mannite, inulin, glycerol, or raffinose. The first three sugars constantly inhibited hemolysis with discoloration of the media after 24 to 48 hours incubation, but lactose and salicin were more inconstant in action and some degree of cell destruction was observed at times without apparent cause. The typical green colony attributed to the pneumococcus or *Streptococcus viridans* was not observed. The remaining four substances had little effect on the size of the hemolyzed zone. Rarely the edges of the decolorized areas were not so sharp as on plain blood agar plates. It was apparent that the sugars which were easily fermented inhibited hemolysin formation most (Lyall (4)), but the resistant carbohydrates occasionally gave uncertain reactions. It is among this last group that the majority of sugars employed for the differentiation of various strains occurs; hence the appearance of the colonies on sugar blood agar plates does not seem accurate as a test of fermentation (Davis (5)). This inhibition of lysin production occurs in liquid as well as solid media. Sekiguchi (6) noted that distinct hemolysin was found only in plain broth, seldom in a weak content of glucose, and rarely in 3 per cent. The degree of inhibition is necessarily dependent to a large extent on the enrichment of the media with serum, since in simple media which are not suitable for the development of a strong hemolysin no trace is evident when an easily fermented sugar has been added previous to inoculation. In the following tests active hemolysin could always be obtained in the presence of carbohydrates.

Experiment 1.—Two flasks of media of 275 cc. volume, identical in every respect except that 1 per cent dextrose had been added to one, were inoculated with equal amounts of a 20 hour culture of Strain A. Bacterial counts, hemolysin determinations, and the hydrogen ion concentration were done at once and at intervals during a 48 hour period. The results are shown in Table I and further illustrated in Text-fig. 1.

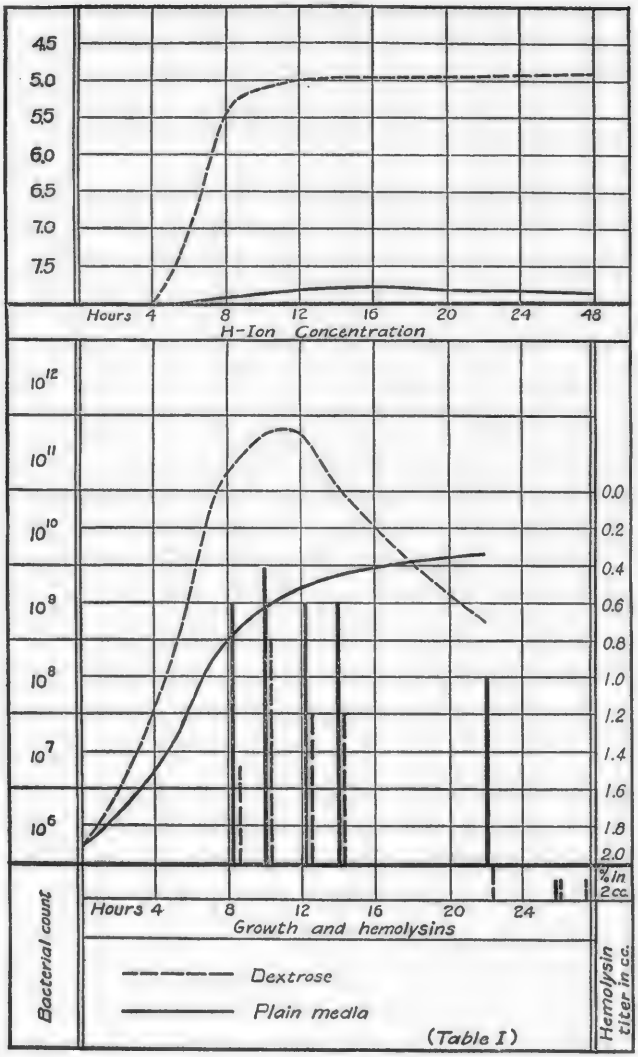
TABLE I.

Interval.	pH	Quantity of fluid.	Hemolysis.*	Bacterial count.†
		Plain media.		
hrs.		*cc.*	*per cent*	
0	8.0	2.0	0	$1.6(10^6)$
4	8.0	2.0	0	$1.9(10^7)$
6	8.0	2.0	0	$2.0(10^8)$
8	7.9	0.6	100	
10	7.9	0.4	100	
12	7.8	0.6	100	$6.5(10^9)$
14	7.8	0.6	100	
22	7.8	1.0	100	$1.1(10^{10})$
28		2.0	50	
48	7.9	2.0	0	
		Dextrose media.		
0	8.0	2.0	0	$1.6(10^6)$
4	8.0	2.0	0	$9.9(10^7)$
6	7.1	2.0	0	$3.8(10^9)$
7	6.3			
8	5.5	1.5	100	$2.1(10^{11})$
10	5.1	0.8	100	
12	5.0	1.2	100	$7.8(10^{11})$
14	5.0	1.2	100	$1.0(10^{11})$
22	4.9	2.0	100	$2.6(10^9)$
28	4.9	2.0	50	
48	4.9	2.0	50	

* Hemolysins are indicated by the amount of supernatant fluid required to hemolyze 1 cc. of the red cell suspension or in the percentage of hemolysis if incomplete.

† The counts are given in powers of 10.

Experiment 2.—The previous experiment was repeated with three flasks, one of plain horse serum beef infusion media, one with 1 per cent dextrose, and one with dextrose and 1 per cent calcium carbonate. These were inoculated with



TEXT-FIG. 1. Comparison of the effects of plain media and dextrose media on lysin production.

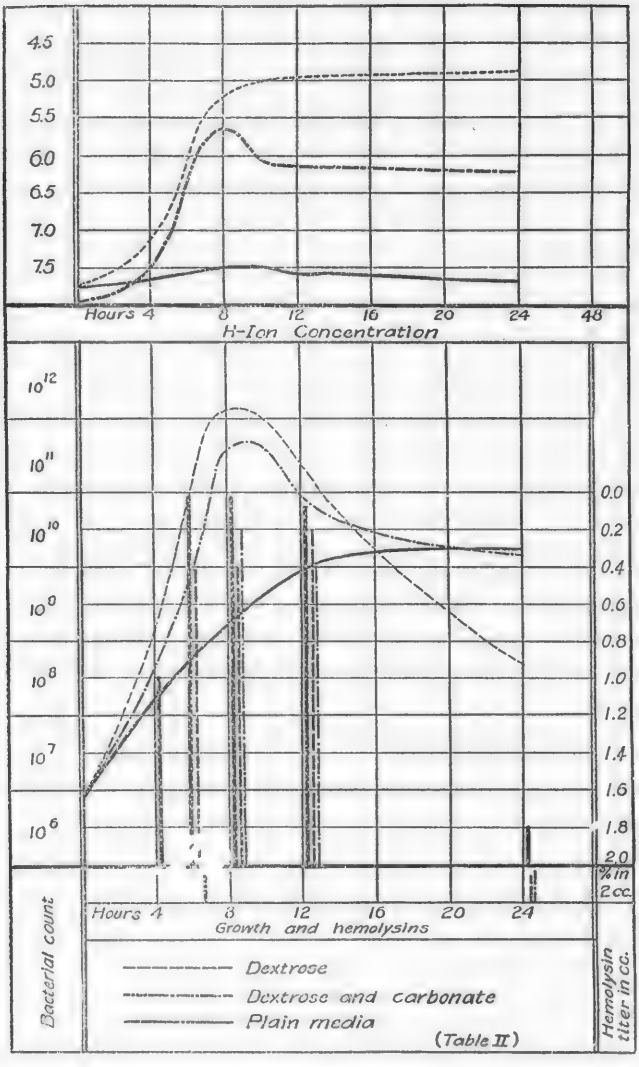
equal amounts of a 20 hour culture of Strain B. The results are illustrated in Table II and Text-fig. 2.

TABLE II.

Interval.	pH	Quantity of fluid.	Hemolysis.	Bacterial count.
		Plain media.		
hrs.		*cc.*	*per cent*	
0	7.8	2.0	0	$8.7(10^6)$
4	7.7	1.0	100	$2.8(10^8)$
6	7.6	0.05	100	
8	7.5	0.05	100	$2.9(10^9)$
10	7.5			
12	7.6	0.1	100	$1.0(10^{10})$
24	7.7	1.8	100	$1.8(10^{10})$
		Dextrose media.		
0	7.8	2.0	0	$8.7(10^6)$
4	7.3	2.0	0	$1.3(10^9)$
6	6.1	0.4	100	
8	5.3	0.4	100	$1.2(10^{12})$
10	5.0			
12	5.0	0.2	100	$4.1(10^{11})$
24	4.9	2.0	50	$5.7(10^8)$
		Dextrose carbonate media.		
0	8.0	2.0	0	$8.7(10^6)$
4	7.5	2.0	0	$5.7(10^8)$
6	6.3	2.0	75	$1.5(10^{10})$
8	5.6	0.2	100	$7.0(10^{11})$
10	6.2			
12	6.2	0.4	100	$6.2(10^{10})$
24	6.1	2.0	75	$1.0(10^{10})$

Experiment 3.—Experiment 2 was repeated, beginning with media titrated to pH 7.6. The flasks were inoculated with equal quantities of a 20 hour broth culture of Strain B (Table III).

The growth of streptococci in dextrose media is much more rapid and profuse than in plain broth, and concomitant with this growth there is a rapid change in hydrogen ion concentration. In cultures which are not so heavily seeded as were those in Tables I to III the maximum acidity is reached within 24 hours (Avery and Cullen (15)).



TEXT-FIG. 2. Comparison of the effects of plain media, dextrose media, and dextrose carbonate media on lysin production.

TABLE III.

Interval.	pH	Quantity of fluid.	Hemolysis.	Bacterial count.
		Plain media.		
hrs.		*cc.*	*per cent*	
0	7.6	2.0	0	$9.2(10^6)$
3½	7.6			
4				$3.7(10^8)$
5	7.5	1.2	100	
6	7.2	0.2	100	
8	7.3	0.1	100	$3.6(10^{10})$
11	7.3	0.05	100	
12				$4.6(10^{10})$
24	7.3	1.0	100	$6.8(10^{9})$
		Dextrose media.		
0	7.6	2.0	0	$9.2(10^6)$
3½	7.6			
4				$2.4(10^8)$
5	6.6	2.0	0	
6	5.5	1.5	100	
8	5.0	0.4	100	$5.0(10^{11})$
11	4.9	0.3	100	
12				$1.9(10^{12})$
24	4.9	2.0	75	$3.2(10^9)$
		Dextrose carbonate media.		
0	8.0	2.0	0	$9.2(10^6)$
3½	8.0			
4				$9.5(10^8)$
5	7.0	2.0	0	
6	6.1	2.0	50	
8	5.5	0.6	100	$5.8(10^{13})$
11	5.5	0.2	100	
12				$2.7(10^{10})$
24	6.4	2.0	50	$5.1(10^{10})$

The greatest increase occurs in the first few hours of incubation during the time that the hemolysin is produced. As the limiting acid concentration is approached the streptococci are found to be less viable, and from that time there is a gradual reduction in the count. Cultures in plain serum broth show a stronger hemolysin

titer during this period of active growth, while the production of hemolysin not only begins earlier but the cultures are actively hemolytic for a longer time. In general, the hemolysin curves obtained approximate the figures of Lyall (4), M'Leod (12), and Besredka (16). The addition of calcium carbonate allows a pH intermediate between those of flasks of plain serum bouillon and of flasks to which glucose alone has been added. With this partial neutralization the growth approaches or exceeds that in dextrose, but the production of hemolysin is still further delayed. In several experiments it was found that the rate of growth in the carbonate dextrose media was inverse to the hemolysin production, and, furthermore, in the cultures without sugar, although there were fewest streptococci, the hemolytic property was always greatest. It is apparent from Tables I to III that with the increased utilization of dextrose the hemolysin production is

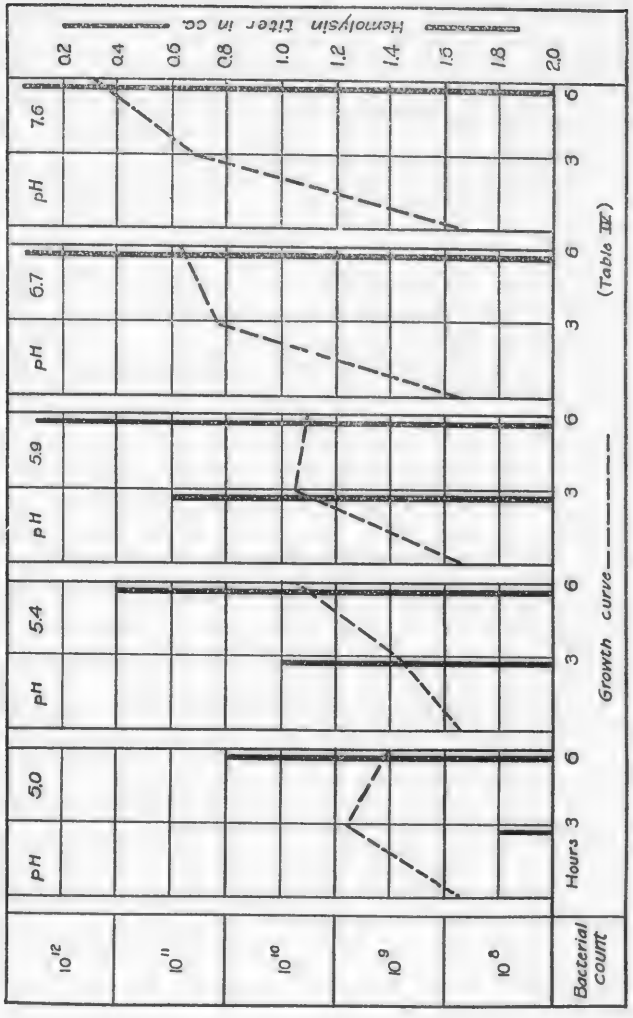
TABLE IV.

pH	3 hrs.		6 hrs.	
	Titer.	Bacterial count.	Titer.	Bacterial count.
	cc.		*cc.*	
7.6		$2.6(10^{11})$	0.05	$1.7(10^{12})$
6.7		$1.1(10^{11})$	0.05	$3.8(10^{11})$
5.9	0.6	$3.0(10^{10})$	0.1	$2.1(10^{10})$
5.4	1.0	$4.0(10^{9})$	0.4	$3.7(10^{10})$
5.0	1.8	$8.8(10^{9})$	0.8	$5.0(10^{9})$

inhibited because of the protein-sparing action of the carbohydrate. The effects of the acids developed in the cultures still further explain these figures.

It was found that when the hydrogen ion concentration of the media was increased before inoculation by the addition of lactic acid, the growth of the streptococci and the hemolysin produced were proportional to the pH. This interferes in no way with the previous findings, since in the curves established the multiplication of the bacteria was not retarded in the first few hours of incubation, yet less lysin was produced. The effect of the acid in Table IV is primarily one of growth inhibition.

Experiment 4.—Five 100 cc. flasks of veal infusion horse serum media were used. The original titer of the media was pH 7.6. Four of the flasks were



TEXT-FIG. 3. The effect of acid on lysin production.

titrated to pH 6.7, 5.9, 5.4, and 5.0 respectively with 0.5 N sterile lactic acid. The volumes were then made equal with sterile 0.85 per cent sodium chloride solution. Each of the flasks was inoculated with the washed streptococci centrifuged from 20 cc. of an 18 hour culture of Strain B. Counts and hemolysin determinations were made after 3 and 6 hours incubation (Table IV, Text-fig. 3). The pH had not changed at the end of the period. Hemolytic titers are designated by the smallest amount of supernatant fluid which would completely dissolve 1 cc. of a 5 per cent suspension of human cells in 1 hour at 37°C. None of the flasks was hemolytic at the beginning. The counts before incubation were 8.5 (10^8).

The effects of acid are still more far reaching. Sterile hemolytic filtrates incubated in various strengths of acid for 6 hours lose a large part of their hemolytic property. That there is destruction of the hemolytic substance is evident, since after titration back to the original pH with sodium hydroxide the solution of the red cells is still less than that of the control tubes. The percentages of hemolysis in the tubes containing the lowest amounts of filtrate serve best as a means of checking this destructive action. In the determinations of hemolysis by the addition of 1 cc. of a 5 per cent suspension of blood cells in salt to small amounts of acid filtrate, the pH is raised to a point which in itself is not destructive to red cells. The acidity in the larger quantities is sufficient to account for the greater hemolysis in the tubes incubated at pH 5.0 for 6 hours. Supernatant fluids acidified and titrated back to neutrality with secondary sodium phosphate after incubation gave similar results. Braun's (13) tables also show this point.

Experiment 5.—An 18 hour culture of Strain B was found actively hemolytic. It was filtered through a new Mandler filter and divided into three equal portions. One was left at its original pH, 7.4. The others were adjusted to a pH of 5.0 and 6.0 by the addition of sterile 25 per cent lactic acid. The hemolytic titer was determined at once and after 3 and 6 hours incubation (Table V). At the end of 6 hours the flasks were neutralized (7.4) and the volumes were made up by the addition of normal salt solution so as to contain an equal concentration of the original filtrate. Cultures of the flasks at the beginning and at the end of the experiment were sterile.

In Table V it was assumed that some of the hemolysis in the strongly acid tubes was due to acid concentration. It was observed that salt solution, to which acid had been added until a pH was reached which

would check the growth of streptococci, had no effect on red cells. If red cells were incubated 1 hour in media made to contain a large quantity of weakly ionized electrolytes and titrated the same way with lactic acid, the solution and discoloration were striking. The destruction of corpuscles begins at about pH 6 and in broth is complete

TABLE V.

Quantity of filtrate.	Hemolysis.		
	pH 5.0	pH 6.0	pH 7.4
Hemolysis before incubation.			
cc.	*per cent*	*per cent*	*per cent*
0.05	100	100	100
Hemolysis after 3 hrs. incubation.			
0.8	100	100	100
0.4	98	100	100
0.2	96	100	100
0.1	78	98	99
0.05	54	96	98
Hemolysis after 6 hrs. incubation.			
2.0	99	87	99
1.0	98	87	98
0.6	84	84	98
0.2	50	68	92
0.1	40	59	86
0.05	30	43	85
Titrated back to pH 7.4 after 6 hrs.			
1.5	28	11	99
1.0	0	22	98
0.8	0	7	97
0.4	0	1	94
0.2	0	0	92

at pH 5. Coincident with this acid hemolysis the hemoglobin is turned brown. Due to the fact that there is so much cell destruction it is impossible to determine the effect of hemolysin on blood cell suspensions which have been previously treated with acid. Lyall (4) has already noted that cells which have been discolored by actively

TABLE VI.

	Control.	0.5 per cent.	1 per cent.	2 per cent.
Filtrate	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.
Sterile sugar solution (10 per cent)	0.0 "	0.05 "	0.1 "	0.2 "
Saline solution	0.9 "	0.85 "	0.8 "	0.7 "
Hemolysis before incubation.				
	per cent	*per cent*	*per cent*	*per cent*
Dextrose	99	99	98	98
Mannite	100	97	97	96
Invert sugar	100	99	99	99
Hemolysis after 3 hrs. incubation.				
Dextrose	97	92	81	82
Mannite	96	95	90	82
Invert sugar	98	96	98	94
Hemolysis after 6 hrs. incubation.				
Dextrose	95	84	61	64
Mannite	87	76	70	61
Invert sugar	90	88	88	83

*Series with Large Quantities.**

Quantity of filtrate.	Control tube.	Dextrose.	Mannite.	Invert sugar.
Hemolysis after 3 hrs. incubation.				
cc.	*per cent*	*per cent*	*per cent*	*per cent*
0.8	100	100	100	100
0.6	99	100	99	99
0.2	99	100	97	98
0.05	97	97	95	96
Hemolysis after 8 hrs. incubation.				
2.0	95	96	95	97
1.0	90	93	90	95
0.6	85	87	86	89

*Cultures were sterile after incubation.

growing pneumococci are more resistant to streptolysin, but this action of the pneumococcus, mentioned by Cole (10), probably enters very little into the discoloration of hemoglobin in dextrose cultures of the beta type streptococcus.

The presence of either dextrose, mannite, or invert sugar exerted a slight inhibition on hemolytic filtrates. This cannot account for any of the marked differences in the titer of broth cultures or in the gross appearance of blood agar plates, but it is probably the cause of the hazy edges occasionally observed in the hemolyzed zones on solid media containing unfermented sugars. It is impossible to demonstrate this effect on large quantities of filtrate, but if the minimal hemolytic amount is incubated in 1 per cent of these sugars the solution of the red cells is much retarded after a few hours (Table VI).

Experiment 6.—The effect of sugars on hemolytic filtrates was studied in the following way. It was found that 0.05 cc. of a sterile filtrate obtained from a 12 hour culture of Strain C caused complete hemolysis of 1 cc. of the red cell suspension. A series of tubes was set up containing 0.1 cc. of the filtrate in 1 per cent dextrose, mannite, and invert sugar; the tubes were incubated 3 and 6 hours. The hemolysin tests are indicated in Table VI. To determine the effect of these substances on larger quantities of filtrate, 50 cc. lots were treated in the same manner and the titer was determined after 3 and 8 hours.

SUMMARY.

It is evident that there are numerous interacting factors which affect streptolysin in the presence of fermented sugars. The principal action is the change in the metabolism of the streptococcus by which more carbohydrate and less protein is utilized; although the growth is much increased there is proportionately less hemolysin. The acid developed in these cultures not only lessens the vitality of the growing organism and so lessens proteolysis, but is destructive to hemolysin at incubator temperature. The concentration of the acid produced causes some hemolysis, and a coincident brown discoloration of the hemoglobin. These principles may be applied to both liquid and solid media.

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PFEIFFER'S BACILLUS AND INFLUENZA.

A Serological Study.

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The literature which has grown up in the very short time since the pandemic of influenza in 1918 is already so voluminous that it cannot be abstracted and analyzed in a brief space. Moreover, such an analysis, at the present time, would not be profitable. The methods employed by different bacteriologists in cultivating the influenza bacillus of Pfeiffer have been so diverse that comparison of results is valueless. There is, at present, hardly any difference of opinion that, with an adequate bacteriological technique, Pfeiffer's bacillus is found to be very commonly present in the respiratory tract of persons suffering from influenza and its attendant pneumonia. Moreover, the bacillus is also widespread in the upper respiratory mucous membranes of persons who have not had influenza, and even of persons who have not, so far as is known, been exposed to the epidemic disease.

We may accept the wide prevalence of the so called influenza bacillus as established and admitted and then proceed to the next and more important, because essential, question of the relationship of the bacillus to the symptom-complex influenza. Except in epidemic periods this symptom-complex is not so definite as to enable a sure and prompt diagnosis of influenza to be made; hence the cultivation of the Pfeiffer bacillus in interepidemic periods, from the upper respiratory tract, is not significant, necessarily, of clinical influenza. Possibly, indeed, there is no pathogenic microorganism, except the omnipresent pyogenic cocci, so frequently present in those parts as the Pfeiffer bacillus.

But even though the etiological rôle of the Pfeiffer bacillus in influenza has come to be seriously questioned, the part it plays in the

pathological complex characterizing this severe disease may still call for definition. Fortunately, in respect to this point there are other means available than mere presence in cultures to determine pathogenic action. The study on which this paper is based was begun early in the epidemic. In the meantime other publications bearing on the same topic have appeared. But it would seem that the subject is one on which additional evidence and more varied tests are desirable; hence the presentation of our results.

Culture Media.

Rabbit blood agar was the routine medium used in searching for Pfeiffer's bacillus during life and at autopsy, until Avery's oleate agar[1] became available. For serological work a medium made in the following way proved most suitable. Fresh rabbit blood was boiled for 2 minutes in a water bath, then centrifuged. The resulting clear, pale pink or yellow fluid was found by spectroscopic examination to contain hemoglobin. Two or three drops were added to 5 cc. of melted agar (pH 7.5) and slanted, while 0.5 cc. was added to a tube containing 20 cc. of broth (pH 7.8). Thus a solid medium and a fluid medium were obtained in which Pfeiffer's bacilli grew abundantly, and washing to get rid of blood or serum was not necessary. The bacilli live only 5 to 6 days on this medium, however. To keep stock cultures, blood broth, in which the bacilli remain viable for 6 weeks or more, proved by far the most suitable medium, while for isolation of the organisms the oleate agar was best.

Serological Reactions.

It was clear that for serological study sera from convalescent patients as well as monovalent immune sera experimentally produced were needed. Rabbits weighing 1,600 gm. proved to be best suited for the purpose of immunization. By means of intravenous injections of live Pfeiffer bacilli, in increasing doses, administered every 3rd day, antisera were produced with ten strains, seven isolated during the epidemic and three obtained several years ago.

[1] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

Agglutination.—It was difficult to get even suspensions of Pfeiffer's bacillus suitable for agglutination tests. Blood broth cultures were not sufficiently profuse, and cultures grown on solid medium clumped spontaneously whether washed or not. Centrifuging had the effect of making the bacilli stick together in small clumps which could not be broken up. Finally, bacilli grown on the boiled-blood-centrifugate-agar were suspended in distilled water, while the serum dilutions were made in normal salt solution. The mixtures were kept at 55°C. for 2 hours, then at room temperature over night. At the end of 2 hours no agglutination had taken place; consequently the 18 to 20 hour reading was the only one that could be noted. The controls obtained by this method were fair for most strains, but unsatisfactory for others. Three strains clumped so much spontaneously that they could not be used for agglutination tests. The strains of Pfeiffer's bacillus varied in the degree of their agglutination reactions, as well as in the kind; four were inagglutinable in every serum tested, one agglutinated only in its homologous monovalent rabbit serum in dilutions of 1: 100, while three other strains clumped in 1: 50 dilutions in that serum. The presence of higher agglutinin content for heterologous than for homologous strains was noted for three sera made with strains of Pfeiffer's bacilli isolated from epidemic cases.

One normal rabbit serum was encountered which agglutinated the Pfeiffer bacillus in a dilution of 1: 20.

The serum of five normal human adults did not agglutinate Pfeiffer's bacillus in any dilution, while two other normal sera reacted in dilutions not exceeding 1: 10. Sera from eleven patients were tested during the 2nd week of illness. Five had had a mild bronchopneumonia attending the influenza attack, and the serum from two of these agglutinated Pfeiffer's bacilli in dilutions of 1: 100, while the other three sera did not contain agglutinins for these bacilli in dilutions higher than 1: 40. Sera from six cases of simple influenza without pneumonia contained no agglutinins for Pfeiffer's bacillus in two instances, and reacted in dilutions of 1: 40 in four others.

Agglutination reactions with the bacillus of Pfeiffer were not satisfactory because of the tendency to spontaneous clumping of the organisms. The wide variations in the results recorded in the litera-

ture may depend partly on this difficulty. Fleming[2] studied twenty-one influenza patients and found the agglutinin content of their sera low; only six agglutinated Pfeiffer's bacillus in dilutions higher than 1:32, and of these only one reacted above 1:128, giving a positive reaction in a dilution of 1:1,000. He also found that strains differed in their ability to agglutinate.

Adults who had been inoculated with vaccine made from several strains of Pfeiffer's bacilli developed agglutinins in their blood within 6 to 10 days, but only rarely were these present in dilutions higher than 1:100.

Complement Deviation.—Antigens were made in two ways: (*a*) Cultures grown in blood broth were centrifuged to throw out the blood cells, and the supernatant fluid was pipetted off, heated to 55°C. for 45 minutes, and tested. Against this antigen controls with sterile broth were always done in parallel series.

(*b*) Cultures in boiled-blood-coagulate-broth were centrifuged for 30 minutes and the precipitate was washed twice in salt solution, then resuspended in fresh salt solution and tested. The use of tricresol was discarded because it tended to make the antigens anticomplementary. A standard of turbidity for the suspended washed bacilli was evolved which approximated 10 cc. of salt solution to the precipitate from 25 cc. of broth culture. However, this amount had to be adjusted to the individual growth.

Tests with cultures of varying ages showed that the maximum strength of antigen was obtained in 3 days. The strains of Pfeiffer's bacilli varied in their antigenic power. In seven instances the antigen was strong, in eight, weak; while seven other strains failed to yield an active antigenic product. Whenever possible, sera were tested against antigen made from (*a*) a meningeal strain isolated during the epidemic, and (*b*) another meningeal strain isolated 2 years ago; (*c*) three respiratory epidemic strains; (*d*) a sporadic respiratory strain; (*e*) a strain isolated from a healthy carrier. Blood from forty-four persons was tested.[3] Of these, fourteen were patients convalescent 6 to 23 days after the onset of an attack of influenza, nineteen

[2] Fleming, A., *Lancet*, 1919, i, 138.

[3] I am indebted to Dr. Walter W. Palmer for his courtesy in allowing me to obtain blood from several patients in the wards of the Presbyterian Hospital.

had recovered from influenza 1 to 4 months previously, four were healthy carriers who had never been ill with influenza, and four were healthy controls. One fatal case of influenzal meningitis was studied, and also two patients who had suffered from diseases other than influenza—lobar pneumonia in one instance, laryngitis and pharyngitis in the other.

Sera from four normal adults were tested as controls. They had not been ill during the past fall and winter, had not suffered from colds, had not been vaccinated against influenza, and carried no Pfeiffer's bacilli in their sputum. No complement-binding reaction was obtained with any antigen in any of these four sera.

Sera from convalescent patients were examined at the end of the 1st week and reexamined in three cases at the end of the 2nd week after onset. Dilutions of 1: 5, 1: 10, and 1: 20 were used and showed a diminishing strength of reaction. The results are shown in Table I. All the sera gave a fixation reaction on the 6th or 7th day, and all but two reacted with antigens made from more than one strain of Pfeiffer's bacillus. The three sera which were obtainable a second time reacted more strongly at the end of the 2nd week than on the 6th day. Five were mild cases without any signs of broncho-pneumonia, while seven were complicated by pneumonia. It is to be noted that the sera of patients who suffered from pneumonia complicating influenza had stronger complement-binding power than did the sera of uncomplicated cases. No convalescent case failed to show fixation antibodies at the end of a week, though these were sometimes small in amount and limited in kind. No differentiation of strains was possible from these results.

One patient had been inoculated 5 weeks before she became ill with influenza with a vaccine of Pfeiffer's bacillus only. Her serum gave a reaction with but one antigen of five used. The influenzal attack had been a mild one, and apparently the inoculation did not increase the complement-binding power of her serum in the presence of Pfeiffer bacillus antigen.

An infant with Pfeiffer bacillus (influenzal) meningitis, who died on the 7th day of illness, gave a strongly positive reaction on the 6th day, with two antigens, one made from a meningeal strain of Pfeiffer's bacillus isolated 2 years before, and the other made from a strain

TABLE I.

Complement Fixation with Blood of Convalescent Patients.

Individual No.	Vaccine.	Day of illness.	Serum dilution.	Antigens.							
				M	D	325	R	W	T	I	280
1	1 mo. before.	9th	1: 5	0	+++	0		0			0
			1:10	0	+++	0		0			0
			1:20	0	+	0		0			0
2	0	9th	1: 5	+++	+++	+++		+++			0
			1:10	+++	+++	++		++			0
			1:20	+	++	+		0			0
3	0	7th	1: 5	0	+	++	++	++	++	++	++
			1:10	0	+	++	+	+	++	+	0
			1:20	0	0	+	0	0	0	0	0
	0	13th	1: 5	+	+++	+++	+++	+++	+++	+++	++
			1:10	0	+++	+++	+	++	++	+	0
			1:20	0	++	++	+	+	0	0	0
4	0	6th	1: 5	0	+	0	0	0		0	0
			1:10	0	+	0	0	0		0	0
			1:20	0	0	0	0	0		0	0
	0	12th	1: 5	0	+++	0	0	0		0	0
			1:10	0	+++	0	0	0		0	0
			1:20	0	++	0	0	0		0	0
5	0	6th	1: 5	+	++	0		0		0	0
			1:10	0	+	0		0		0	0
			1:20	0	0	0		0		0	0

W is a strain of Pfeiffer's bacillus isolated from a case of influenzal meningitis during the epidemic; T a strain isolated from a case of influenzal meningitis 2 years ago; M and D strains isolated from the lungs of influenzal pneumonia cases during the epidemic; 325 and R strains isolated from the sputum of influenzal pneumonia cases during the epidemic; I a strain isolated from the lungs of an influenzal pneumonia case 3 years ago; and 280 a strain isolated from the throat of a healthy adult carrier.

0 indicates complete hemolysis, no fixation of complement; + + + no hemolysis, complete fixation of complement; + + and + varying degrees of fixation of complement.

TABLE I—*Concluded.*

Individual No.	Vaccine.	Day of illness.	Serum dilution.	Antigens.							
				M	D	325	R	W	T	I	280
5	0	12th	1: 5	++	+++	0		0		0	0
			1:10	0	+++	0		0		0	0
			1:20	0	+	0		0		0	0
6	0	7th	1: 5	0	+++	++	+++	+++			
			1:10	0	+++	++	+++	+			
			1:20	0	++	0	+	+			
7	0	8th	1: 5	0	+++	+++	+++	+++			
			1:10	0	+++	++	+++	+			
			1:20	0	+++	0	+	0			
8	0	14th	1: 5	0	+++	+++	0	+++			
			1:10	0	+++	+	0	+			
			1:20	0	+	0	0	+			
9	0	6th	1: 5			+++			++		
			1:10			+++			++		
			1:20			+			+		
10	0	9th	1: 5	+	+++	++	++	+++	+++	++	++
			1:10	0	+++	++	+	+++	+	+	+
			1:20	0	+	0	0	++	0	0	0
11	0	7th	1: 5		+++					+	
			1:10		+					+	
			1:20		0					0	
12	0	14th	1: 5	++	+++				++	++	
			1:10	++	+++				+	+	
			1:20	0	+++				0	+	
13	0	13th	1: 5		+++					++	
			1:10		+++					+	
			1:20		++					+	
14	0	23rd	1: 5	+++	+++	+++	++		++	++	++
			1:10	++	+++	+++	+		++	++	+
			1:20	+	+	++	+		++	++	+

recovered from the lung in a fatal case of bronchopneumonia occurring during the epidemic. With antigen of a strain of Pfeiffer's bacillus from a healthy carrier, isolated during the course of the epidemic, and with that of a respiratory strain isolated from a sporadic case 4 years ago, no reactions were obtained. It is to be regretted that it was not possible to obtain a larger quantity of serum from this baby, so that it might have been tested with antigen made from its homologous strain of Pfeiffer's bacillus. The results obtained with this serum confirm the point previously shown;[4] namely, that meningeal strains of Pfeiffer's bacillus are virulent and yield a strong antigen, and that while respiratory strains are more apt to be nonvirulent and yield a weak antigen, virulent respiratory strains may be encountered which yield a strong antigen.

Complement fixation tests with Pfeiffer bacillus antigen made with the blood of nineteen individuals who had entirely recovered from influenza, which had attacked them 1 to 4 months previously, gave irregular results. At the end of a month one serum gave only an incomplete reaction with two antigens, and after 6 and 7 weeks only low complement-binding content was found in two other sera. 2 months after the illness no reaction was obtained with one serum, while three others gave strong reactions even in dilutions of 1: 20, and a fourth reacted well in a dilution of 1: 5, but not higher. After 3 months one serum gave complete reactions only in dilutions of 1: 5, while another was positive in dilutions of 1: 10. 4 months after influenza two sera were entirely devoid of complement-binding body content.

1 month after the influenzal illness a man who had been inoculated with three doses of vaccine made with three strains of Pfeiffer's bacillus, 4 months before the attack began, gave very strong reactions in dilutions of 1: 10. 2 months after the attack of influenza the sera of two subjects inoculated with Pfeiffer bacillus vaccine 3 months before the illness reacted as strongly as did two unvaccinated patients, while one reacted much less well. In comparing the serum of inoculated and of non-inoculated individuals, it becomes evident that the administration of a vaccine, made from several strains of Pfeiffer's

[4] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

bacillus, did not have any apparent influence on the degree of the complement-binding reaction with the antigens of Pfeiffer's bacillus when an interval of 3 or 4 months had elapsed between the inoculation and the influenzal illness. The patient whose serum was poor in this fixing antibody 1 month after the attack is apparently the exception, when viewed in the light of Table I.

Sera from four carriers were tested. All denied any respiratory illness in the previous 6 months. One had been inoculated with vaccine made from Pfeiffer's bacilli only. Two of the four carriers gave no reaction with several antigens. The inoculated individual and one person who had not been vaccinated reacted strongly. One of the carriers whose serum was negative to this test received Pfeiffer bacillus vaccine, and 1 week after the third dose had been injected his serum contained complement-binding bodies for three of six antigens against which it was tested.

To sum up, complement-binding antibodies were absent from the blood of four normal individuals who had not had influenza, and who were not carriers of Pfeiffer's bacillus. In the blood of influenza patients the fixing antibodies were present at the end of the 1st week, increasing in strength during the 2nd week, and were, as a rule, demonstrable at the end of 3 or 4 weeks. At the end of the 2nd month after an attack of influenza these antibodies were strongly present except in one case. At the end of the 3rd month complement fixation was still demonstrable in patients' serum, but in 4 months it had entirely disappeared. A complicating pneumonia increased the complement-binding power of the serum.

Monovalent immune rabbit sera were made with ten strains of Pfeiffer's bacillus and all bound complement in the presence of antigens made both from homologous and from heterologous strains. Normal rabbits were used as controls and their sera gave negative results.

Precipitins.—With the same antigen as that used for the complement fixation test, precipitins were demonstrable in every convalescent patient's serum tested, while they were absent from the serum of normal persons. Antigens made from homologous strains of Pfeiffer's bacillus gave no stronger precipitin reactions than did those made from heterologous strains. The earliest precipitin reaction noted was on the 6th day of the illness, and the latest, 3 months after the attack.

Poisonous Filtrates.

According to Parker[5] filtrates of Pfeiffer bacillus cultures grown in heated blood broth for 6 to 20 hours are poisonous for rabbits when inoculated intravenously. Tests were made with twenty-five strains of the bacilli. The filtrates from seven strains killed rabbits weighing 1,300 to 1,600 gm. within 1 to 2½ hours when inoculated intravenously in doses of 2 or 3 cc. Much larger doses (5 cc.) were required to kill the animals with filtrates of other strains. Death was sometimes, though rarely, delayed until 12 to 15 hours after the injection of the poison.

The effects of intravenous injection of a potent filtrate from an early culture of Pfeiffer's bacillus were quite uniform. The animals gradually became more and more quiet, so that in 30 to 45 minutes they sat in a hunched up position with no movement but that of very rapid respiration. Intestinal peristalsis was increased until at the end of 60 to 90 minutes the animals were passing fluid feces. Gradually the head leaned, then fell to one side, and the animal slowly fell over. After one or two attempts to rise, all muscular efforts ceased, and it lay on its side, breathing very rapidly and irregularly, with the head becoming more and more retracted. Death was often preceded by general convulsions. Non-lethal doses caused the symptoms of muscular weakness, irregular respiration, retraction of the head, and diarrhea; but the rabbit did not fall over on its side or go into a convulsion, and recovery gradually took place so that the animal was well within 3 or 4 hours, though more quiet than before the injection. The leucocytes fell within 1 hour after inoculation, and a difference of 5,000 to 12,000 from the initial count was noted. The recovered animals showed a rise within 4 to 5 hours, and on the following day the leucocytes were always as high or higher than before the injection of the filtrate.

Postmortem examination showed constant changes in the lungs. These organs were deep pink in color, mottled with dark red or brown areas of hemorrhage, often most marked in one or both lower lobes. Punctate subpleural hemorrhages were numerous and general. The lungs were edematous; frothy fluid exuded from the cut surface and

[5] Parker, J. T., *J. Am. Med. Assn.*, 1919, lxxii, 476.

from the trachea and bronchi. The mucosa lining the larynx, trachea, and large bronchi was dark red or purple in color from an intense congestion, while bright red points of capillary hemorrhages were numerous.

The intestinal contents were fluid. The kidneys and liver were congested. In female rabbits the Fallopian tubes were always bluish red in color and on section showed an intense congestion of all the coats. The blood vessels in the cerebrospinal meninges were filled with blood. No gross hemorrhages were apparent in the nervous system. The suprarenals were pale pink in color.

Microscopic examination of the brain showed minute areas of perivascular diapedesis in sections of the medulla. The lungs showed a congestion of all the vessels so exquisite as to constitute a natural injection. A comparative absence of leucocytes was striking; both within the lumen of the vessels and in the alveoli they were few in number. The connective tissue septa were edematous. Many alveoli contained red blood cells, and the epithelium lining the walls was intact; other alveoli contained granular, coagulated serum in which air bubbles were apparent.

In the female the Fallopian tubes showed an intense congestion of the blood vessels in the mucosa, while some muscle fibers had undergone hyaline degeneration.

Control experiments were made by injecting normal incubated broth intravenously into rabbits. No symptoms followed, and at autopsy no lesions were found. A filtrate of typhoid bacillus culture was also injected as control. The animals died after 15 to 18 hours, and at autopsy showed a hypostatic congestion of the lungs and some discoloration due in large part to the fact that they had been dead several hours before they were examined. The edema and punctate hemorrhages were lacking.

The sera of rabbits inoculated with increasing doses of filtrates of young cultures of Pfeiffer's bacilli occasionally protected other rabbits against one fatal dose of homologous and heterologous filtrates, but no serum protected against more than one lethal dose of poison. Normal rabbit serum protected almost as many animals as did the sera of the inoculated rabbits. The nature of the poison remains in doubt, but it is evidently not a toxin, since there is no definite incu-

bation period between its inoculation and the appearance of symptoms, and a protective serum does not result from its repeated injection into rabbits.

Protection Experiments.

Attempts to protect mice against lethal doses of Pfeiffer's bacilli by means of rabbit sera made with poisonous filtrates proved unsatisfactory. The sera never reached a high potency because the rabbits became emaciated and died when the injected poison was a strong one, and they reacted but slightly to a weak one. Consequently the results of protection experiments on mice with such a serum were irregular and misleading. A strong antibacterial serum was more readily obtained and protected a greater number of mice against a lethal dose of Pfeiffer's bacilli, whether the serum was given 24 hours before the bacilli were injected, or after 15 minutes contact with the dose of culture (Table II).

TABLE II.

Protection Experiments with Mice.

No. of animals.	Serum (rabbit).	Method of administration.	Survived.
			per cent
53	Antitoxic (?).	24 hrs. before bacilli.	3.8
34	" (?).	After 15 min. contact with bacilli.	23.0
16	Antibacterial.	" 15 " " " "	25.0
20	"	24 hrs. before bacilli.	40.0
10	Normal.	24 " " "	1.0
10	"	After 15 min. contact with bacilli.	2.0

Human sera from six recovered patients were entirely lacking in protective effect when tested on mice inoculated with lethal doses of Pfeiffer's bacillus, although all these sera contained complement-binding bodies.

DISCUSSION.

As regards the serological reactions of Pfeiffer's bacillus with the sera of recovered patients, as well as with monovalent immune rabbit sera, the results with agglutination were irregular and not satisfactory.

As far as they showed positive results the epidemic and the sporadic strains reacted similarly.

At the Department of Health of the City of New York, Park and his coworkers[6] found Pfeiffer's bacillus present in 80 to 100 per cent of influenza patients. The bacilli isolated from different cases, however, did not produce identical immune bodies in inoculated animals as measured by the agglutinin absorption test, and Park concludes from these studies that there were many strains of Pfeiffer's bacillus, and not one epidemic strain, present during the pandemic.

Complement fixation reactions with human sera showed that normal controls did not contain complement-binding bodies for the Pfeiffer bacillus, but that the blood of recovered patients gave a positive reaction with more or less regularity in dilutions varying from 1:5 to 1:20. The binding could almost always be obtained with antigens made from more than one strain of the bacillus. On the other hand, with the sera of two patients in whose sputum Pfeiffer's bacilli were not found, and who suffered from lobar pneumonia and laryngitis respectively, no fixation was obtained. It would seem, then, that the reaction is due to something more than the presence of an increased amount of non-specific protein after a febrile attack.

With some immune rabbit sera binding was obtained in dilutions of 1:100. Normal human and rabbit serum controls did not bind.

Rapoport[7] examined a much larger series of cases than I was able to do. He studied influenzal pneumonia patients and controls. His results showed complement fixation bodies in 54.5 per cent of 295 convalescent influenzal pneumonia patients and in only 9.67 per cent of 300 controls. My results confirm his and show that patients who are convalescent from influenza without pneumonia also may have complement fixation bodies in their serum for a limited period of time and to a less marked degree.

Kolmer, Trist, and Yagle[8] demonstrated complement fixation in the serum of convalescent influenza patients with antigens made from Pfeiffer's bacillus, hemolytic streptococci, and *Micrococcus catarrhalis* but not with staphylococcus and pseudodiphtheria bacillus antigens.

[6] Park, W. H., *J. Am. Med. Assn.*, 1919, lxxiii, 318.
[7] Rapoport, F. H., *J. Am. Med. Assn.*, 1919, lxxii, 633.
[8] Kolmer, J. A., Trist, M. E., and Yagle, E., *J. Infect. Dis.*, 1919, xxiv, 583.

45 to 50 per cent of the sera reacted with Pfeiffer's bacillus, and only 38 per cent bound complement when streptococcus or *catarrhalis* antigens were employed. All the sera were tested in a dilution of 1:10. Consequently no light is thrown on the specificity of the reactions.

Precipitins were also found constantly in the sera of recovered patients and of immunized rabbits. The reaction for them was strongly marked both with heterologous and with homologous strains of Pfeiffer's bacillus.

CONCLUSIONS.

It has been shown that the sera of patients convalescent from influenza yield reactions for agglutinins, precipitins, and complement-binding bodies with antigens of Pfeiffer's bacillus. These reactions appear constantly at the end of the 1st week, increase in intensity during the 2nd week, and remain demonstrable for a period of 2 to 4 months. They were most complete in the sera of patients suffering from postinfluenzal pneumonia. It has also been demonstrated that the strains of Pfeiffer's bacillus isolated during the epidemic were morphologically and biologically similar to the strains isolated from influenza cases in other years, and antigenically they differed from them only quantitatively. The patients' serological reactions indicate the parasitic nature of the bacillus, but are not sufficiently stable and clean-cut to signify that Pfeiffer's bacillus is the specific inciting agent of epidemic influenza. They do, however, indicate that the bacillus of Pfeiffer is at least a very common secondary invader in influenza, and that its presence influences the course of the pathological process.

A STUDY BY THE SINGLE CELL METHOD OF THE INFLUENCE OF HOMOLOGOUS ANTIPNEUMOCOCCIC SERUM ON THE GROWTH RATE OF PNEUMOCOCCUS.

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Ascoli (1) attributed the immunity observed in certain experiments with anthrax to the antimetabolic action of immune serum on the bacilli, represented, in part, by the inhibition of capsule formation. He was the first to use the term antiblastic immunity in this connection. Dochez and Avery (2) found that the earlier growth of pneumococcus in homologous antipneumococcic serum was inhibited by the serum. They estimated the degree of inhibition by plating out samples and counting colonies. They reported a further inhibition of the metabolic activities of pneumococcus by homologous serum as measured by the production of amino-acids and by the fermentation of glucose, saccharose, lactose, and inulin. The serum of patients at different stages of pneumonia infection showed a like inhibitory action on the digestion of protein and the fermentation of inulin. Blake (3) repeated the experiments of Dochez and Avery and extended them. He obtained, in part, similar results, but put a different interpretation on the findings. Since pneumococcus tends to grow in chains and clumps in homologous serum, a sample of such growths would give fewer colonies than the controls, even when the numbers of bacteria were exactly the same. Further, the clumped masses of bacteria tend to collect in the bottom of the test-tube, and the lack of diffusion of formed products would explain the results of Dochez and Avery in regard to the fermentation of sugars and the production of amino-acids. In cultures frequently shaken or grown in shallow layers of medium in flasks Blake obtained the same metabolic activities in homologous serum as in controls. Homologous sera deprived of agglutinins gave the same results as heterologous sera. He confirmed the observation of von Dungern (4) that the liquefaction of gelatin by *Staphylococcus aureus* is inhibited by homologous serum, but, according to his observation this inhibition of liquefaction was not accompanied by a diminution of such metabolic activities as are essential to growth.

It seemed worth while to investigate further the possible inhibition of the growth of pneumococcus by homologous immune serum, with especial reference to the effect of the serum on the earlier genera-

tions, by a method which allows a comparatively accurate measurement of the growth rate of bacteria. The problem of infection and resistance is complex, involving factors the action of which is still obscure; and if one possible factor should be demonstrated and prove to be measurable, or if it should be definitely eliminated, we would have an advance in our knowledge of the mechanism of immunity.

In addition to accuracy in measurement of growth rate, the use of the single cell method offers other advantages in this problem. Similar sowings, the immediate progeny of a single cell, may be employed for the test culture and the controls. Agglutination may be eliminated, in the earlier generations, at least. Further, it is possible that the use of single or few cells in culture experiments may more closely simulate the process of infection in nature.

The method of single cell culture as described previously (5) was employed in these experiments with some improvements and minor modifications to suit the problem and the organism studied. No especial difficulty was encountered in working with pneumococci. They were easily isolated and grew readily, and multiplication in hanging drops proceeded apparently at the same rate as in the test-tube.

In practically all the experiments in hanging drops described in this paper, controls were made under the same conditions, so that it was not necessary to ascertain exactly the normal growth rate of pneumococcus. However, it was found that some approximation of this growth rate would be convenient, especially in estimating the degree of lag, when such occurred. Many tests showed that with Type I and Type II pneumococcus, grown in hanging drops under the best conditions—a favorable medium and temperature, and seeding material taken in an actively dividing stage—the growth rate approximates one generation in 30 minutes. In a few instances growth was apparently more rapid, possibly a generation every 25 minutes, but this occurred in only a small minority of the tests. Single pairs sown in test-tubes of 5 or 10 cc. of serum broth gave approximately the same rate. Here the number of cells in the culture was estimated by means of the counting chamber. It is hardly practicable to estimate the minimum generation time by means of plating out methods, since at the period of growth when the chains are breaking up, one

would get an apparent growth rate much more rapid than the real one. As the author has shown (6), colon bacilli also break up into much smaller elements at a certain period of growth, so that the plating out method with bacilli also is subject to the same error. In this paper the generation time of 30 minutes is taken provisionally as the normal rate for pneumococcus.

Experiments with Immune Horse Serum in Hanging Drops.

The method and results of hanging drop experiments are best illustrated by protocols of one or two experiments.

A single pair of Pneumococcus Type I, grown for some time on artificial media, was sown in a hanging drop of blood broth. After about five generations had formed, single pairs of the progeny of this pair were sown in hanging drops of homologous highly immune horse serum of Type I and also in drops of highly immune horse serum of Type II and of normal horse serum, all diluted 1:52 in nutrient broth. The growth was studied in five droplets of the diluted homologous serum, four of the heterologous serum, and two of the normal horse serum, all on the same large cover-glass and under like conditions. After 170 minutes incubation the cells in the droplets were killed and stained by injecting a stain through a capillary pipette under microscopic control. A special fixing and staining fluid, made by adding medicinal methylene blue to a solution of potassium hydroxide immediately before use, was employed. The number of pairs present was determined with a $\frac{1}{12}$ oil immersion lens, and the size of the organisms in the various droplets was compared and also compared with the size of the seeding pairs which had been noted at the time of inoculation.

In this experiment one pair in heterologous serum failed to grow. In all the remaining ten drops, including those containing the homologous serum, the amount of growth was almost exactly the same—slightly less than six generations—giving a growth rate of approximately 29 minutes. There was no greater tendency to lag in the homologous serum than in the controls.

In the experiment just described conditions were favorable and growth proceeded at a geometrical rate and at approximately the maximum rate for pneumococcus in hanging drops. In a second experiment the conditions were somewhat different. Undiluted serum was used with just enough nutrient agar added to make a thin jelly, two parts of agar to six of serum. In preparing the seeding material a single cell was isolated from a blood broth culture of Pneumococcus Type I, made from the blood of a pneumonia patient, and this cell was grown in a hanging drop of human blood broth to four generations. The cells formed adhered in a chain, and only four generations formed in $2\frac{1}{2}$ hours, indicating that the pair sown was not actively growing at the time of isolation. This chain was broken up and distributed in drops of homologous and heterologous sera as shown in Table I.

TABLE I.

Growth of Pneumococcus Type I in Agar Serum Jelly. Incubation 180 Minutes.

Type I serum.			Type II serum		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	1	4	1	1	4
2	1	3	2	2	4
3	3	3	3	3	3

In this experiment growth evidently lagged and the cells stained irregularly, so that the number of generations could only be approximated, but there was apparently no greater inhibition of growth in the homologous serum than in the control.

The protocols given above are representative of many hanging drop experiments. Sera were used undiluted and in dilutions up to 1:52. Dilutions were made in normal salt solution as well as in plain broth, glucose broth, and agar. Seeding material was taken from cultures long grown on artificial media, from infected human blood, and directly from the heart's blood of mice that had died of pneumococcus infection. In some experiments seeding material was taken at the height of growth, in others from a 22 hour culture, and in others from cultures at various other periods of growth. Lag and irregular growth occurred in some instances, especially when the seeding material was old or when it was sown in undiluted or little diluted sera, but in all these experiments the controls showed the same degree of irregularity and inhibition of growth as occurred in the homologous serum. To sum up, the hanging drop experiments gave no evidence of a measurable inhibition of growth in homologous serum.

While the growth rate in homologous serum was apparently unaffected, the character of the growth was distinctly different from that observed in heterologous serum or in other controls. The cells early became invested with a thick capsule and grew in chains, which often intertwined and formed zooglea-like masses. It was sometimes necessary to disentangle these chains by means of a fine capillary point, in order to count the cells. The capsule formed also on cells which were isolated from old cultures and were apparently dead or, at all

events, subsequently showed no growth in hanging drops. When sputum containing pneumococcus was incubated in a mixture of broth and homologous serum, these zooglea-like masses were distinctly seen, suggesting a possible means of early recognition of types of pneumococcus in sputum. This type of growth can hardly be called agglutination in the strict sense of the word, but resembles zooglea formation. The thick capsules are probably concerned in the protection of the organism, and their formation may be analogous to the formation of capsules by many bacteria in the animal body.

Experiments with Immune Horse Serum in Test-Tubes.

If homologous immune serum causes no inhibition of the growth of pneumococcus during the first five or six generations, it is not likely that any inhibition would occur in later growth, except that in the later periods the clumping of the cells and localization in one part of the container might interfere with the free diffusion of nutrient material and waste products, and in this way interfere with the free growth of the cells. Nevertheless, it seemed important to investigate this matter, and experiments of the following type were performed. One or more pairs of pneumococci were inoculated into test-tubes containing 2 to 10 cc. of diluted sera. In the experiment given in Table II 1 per cent glucose broth was used to dilute the serum, since the clumps formed in homologous immune serum when diluted with this medium apparently disintegrate earlier than when the dilutions are made in plain broth, and the cells can therefore be more readily counted. In this experiment homologous and heterologous antipneumococcic sera were employed in a dilution of 1:26 in 2 cc. of broth. As a control, Tube 7, which contained 11 cc. of glucose broth plus 0.1 cc. of rabbit serum, was included. As seeding material we used the progeny of one pair of Type I pneumococci, grown for 2 hours in a hanging drop of rabbit serum glucose broth. At the end of this period sixteen pairs had formed. The cells were apparently in an active state of multiplication.

The number of bacteria was ascertained by counting the stained cells in a counting chamber. In Tube 1 chains and clumps appeared, so that only an approximate count could be made, but, since even an

error of 100 per cent in counting would make a difference of only one generation, the error in the estimated generation time must be small. The growth in Tubes 3 and 4 was allowed to continue until the medium had become densely cloudy and the clumps had largely disintegrated. A more accurate count could then be obtained. In Tubes 3, 4, and 7, the growth had begun to lag on account of crowding before the count was made, so that the generation time is apparently greater in these tubes. All the cultures were well shaken at intervals after about 12 hours growth. Tube 2, containing Type II serum, showed cloudiness before any other. Otherwise the growth was ap-

TABLE II.

Growth of Pneumococcus Type I in Glucose Broth. Homologous and Heterologous Immune Sera, 1 : 26 Dilution.

Tube No.*	Type of serum.	Sowing. Pairs.	Length of incubation before cloudiness appeared.	Length of time allowed to grow before counting.	No. of generations formed.	Generation time.
			hrs.	*hrs.*		*min.*
1	I	1 medium sized.	$13\frac{3}{4}$	$14\frac{1}{3}$	26.2	32.8
2	II	1 " "	$12\frac{1}{2}$	$14\frac{1}{3}$	27.4	31.4
3	II	1 " "	13	$20\frac{1}{2}$	28.8	42.8
4	I	1 " "	13	$20\frac{1}{2}$	29.1	42.4
5	II	2 small.	No growth.			
6	I	2 "	" "			
7	Rabbit serum.	2 "	16	$18\frac{5}{6}$	30.2	34.3

* The tubes were inoculated in the order given.

proximately the same in all the tubes containing immune serum. In the two tubes containing homologous serum in which growth occurred, the clumping ordinarily observed in such serum was manifest.

In the following experiment (Table III) the homologous and heterologous sera were diluted 1: 10 in plain broth and 5 cc. quantities were used. The seeding material, Type I pneumococcus, was taken directly from a broth culture at the height of growth. The cells were isolated and each was separately transferred to the serum dilutions in the test-tubes. Six hanging drop cultures in plain broth were also made at the same time with cells isolated from the same source. In five of these cultures two generations occurred during

the 1st hour of incubation, and in one, one generation. It is probable therefore, that all cells sown into the serum dilutions were viable and actively growing.

On account of the formation of large clumps in the homologous serum tubes, it was hardly practicable to estimate comparative amounts of growth either by counting or by the degree of cloudiness. In consideration of the smallness of the sowings, it is apparent that growth in the homologous serum suffered little if any lag. The number of successful growths in the homologous serum was nearly as great as that in the heterologous, three out of five in the one and four out of five in the other.

TABLE III.

Growth of Pneumococcus Type I in Plain Broth. Homologous and Heterologous Serum, 1 : 10 Dilution.

Tube No.*	Type of serum.	Sowing. Pairs.	Growth after 15¾ hrs. incubation.
1	I	1	Clear above; clumps at bottom.
2	II	1	Cloudy ++.
3	I	1 small.	No growth.
4	II	1 "	" "
5	I	1 "	" "
6	II	1 "	Cloudy ++.
7	I	2 "	Clear above; clumps at bottom.
8	II	2 "	Cloudy ++.
9	I	4 "	Clear above; clumps at bottom.
10	II	4 "	Cloudy ++.

* The tubes were inoculated in the order given.

In another experiment 10 cc. lots of broth plus homologous serum, diluted 1: 50 and 1: 100, were employed, and the growth in these was compared with the growth in similar quantities of the same broth without any serum. Sowings of one and two pairs were made. There was less lag in the tubes containing serum than in the plain broth tubes, probably entirely due to the presence of the serum. Clumping occurred in the 1: 100 dilution but in less degree than in the 1: 50 dilution. Growth was visible macroscopically in both dilutions at the end of 15 hours, while the plain broth tubes were still clear 3½ hours later.

In short, the test-tube experiments show that growth in homologous serum proceeds at nearly, if not quite the same rate as that in the heterologous serum in spite of the formation of clumps.

Action of Homologous Serum Plus Complement.

In the experiments described above no fresh complement was added other than that contained in blood or serum broth. In order to test the effect of homologous serum plus complement on growth rate, preliminary experiments were arranged in which varying amounts of fresh rabbit blood, fresh human blood, or rabbit blister fluid containing more or less active leucocytes were added to homologous serum. In these mixtures the homologous serum gave no indication of a greater power to restrain growth of pneumococcus than the controls.

Experiments in Vivo.

Intravenous Injection of Immune Serum.—It was then proposed to ascertain whether homologous serum acquired some growth-inhibiting substance through contact with animal tissues *in vivo*.

A normal rabbit, weighing 3,200 gm., was given intravenously 5 cc. of a Type I immune horse serum. 1 hour later the rabbit was bled from the ear. Before coagulation had taken place, part of the blood was diluted in plain broth, one part of blood to four of broth, and another portion was collected in a centrifuge tube, in order to obtain serum for the tests to be described later. To serve as controls, blood of the same rabbit taken immediately before the injection of the serum was similarly diluted, and, in addition, a second control was made by adding 0.5 cc. of the normal rabbit blood to 9 cc. of broth containing 0.5 cc. of the same Type I serum that was inoculated into the rabbit. The tubes were immediately transferred to the ice chest. A series of hanging drop cultures was made at the same time in the 1:5 dilutions of normal blood, in the blood taken after the serum injection, and in the mixture containing normal blood, immune serum, and broth. The inoculation of these drops was made with cells of Pneumococcus Type I taken from a young broth culture. After 2 hours incubation the growth formed was stained on the coverglass and the cells were counted under the $\frac{1}{12}$ oil immersion lens. The results are given in Table IV.

TABLE IV.

Growth of Pneumococcus Type I in Hanging Drops. Normal and Immune Rabbit Blood, Dilution 1:5 in Broth. Incubation 2 Hours.

Normal blood.			Blood after injection of immune horse serum.			Normal blood plus homologous immune horse serum.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	1½	0	1	1	3	1	1	3
2	1	4−	2	2	3	2	3	3
3	2	4	3	2	3+	3	1	3+
4	2	3−	4	1½	0	4	2	3+
5	1	4+	5	1½	0	5	1	3+
6	3½	2+	6	1	1	6	1	3
7	2	3	7	1	4	7	1	2
8	1	3	8	1	1	8	½	0
9			9	1	3	9	2	3

It is seen in Table IV that growth took place in a large proportion of the hanging drops, averaging about three generations in 2 hours. A varying amount of lag is shown, due possibly to unevenness in the sowing material, but, in the aggregate, growth was practically the same, in all the groups. The tendency to capsule formation, characteristic of growth in homologous serum, appeared in all except the controls in normal blood. The experiment was repeated on the same day, with actively dividing cells of like ancestry grown on the cover-glass as sowing material. The same 1:5 dilutions in broth of blood taken before and after immunization were employed, but plain broth was substituted for the control with normal blood plus homologous serum. Growth proceeded at nearly the normal rate for hanging drop cultures —three to four generations in 90 minutes. The rate of growth in the blood of the animal which had received immune serum was practically the same as that in the blood of the normal animal.

On the following day a comparison was made of the undiluted serum of the rabbit taken before and that taken 1 hour after injection of immune serum. Seven hanging drops were made of each, and sowings were made from an actively growing broth culture. The serum was undiluted except through the small amount of moisture of conden-

sation present on the cover-glass. Plain broth controls were used. Growth occurred in all, with the exception of one broth control, but tended to lag in the undiluted serum. There was no measurable difference between the number of generations formed in the normal serum and in that obtained following the injection.

In order to compare the results obtained in hanging drops with those obtained in test-tubes an experiment was arranged with the same sera that were used in the last experiment. The sera were diluted in plain broth, 0.5 cc. of serum to 4.5 cc. of broth. Sowings were taken from a young plain broth Pneumococcus Type I culture, and, in order to get similar cells, one long chain of cocci was broken up to furnish the sowings. The results are given in Table V.

TABLE V.

Growth of Pneumococcus Type I in Test-Tubes. Normal and Immunized Sera, Dilution 1:10 in Broth.

Tube No.*	Rabbit serum.	Sowing. Pairs.	Growth after 15 hrs. incubation.	Growth after 19½ hrs. incubation.
1	After injection of immune serum.	2	Clear above with a clumped mass in the bottom.	Cloudy ++.
2	Before injection of immune serum.	4	Cloudy ++.	" ++.
3	After injection of immune serum.	2	Clear.	" ++.
4	Before injection of immune serum.	2	Cloudy ++.	" ++.

* The tubes were inoculated in the order given.

It will be noted in Table V that growth took place in all the tubes, with some lag in No. 3. However, in consideration of the variability of lag in test-tube experiments of this kind, whether immune serum is present or not, the lag in this tube can hardly be attributed to the presence of the homologous serum.

The experiments with the serum of the passively immunized rabbit were repeated with the serum of another similarly immunized animal, with especial attention to any inhibitory substance in the whole blood or undiluted serum. A 3,000 gm. rabbit was given intravenously 5 cc. of a potent Type I serum. 23 hours later a sample of blood was

drawn from an ear vein into a capillary pipette, and, before coagulation had taken place, was added to sowings previously isolated in minimum sized drops on the cover-glass. The sowings were taken from an actively growing rabbit blood broth culture of Type I pneumococcus. To serve as controls, a similar series of drop cultures was prepared on the same cover-glass with normal rabbit blood and rabbit blood broth. The results after $2\frac{1}{2}$ hours incubation are given in Table VI.

TABLE VI.

Pneumococcus Type I. Culture in Immunized Rabbit Blood, in Normal Rabbit Blood, and in Broth.

Blood of passively immunized rabbit.			Blood of untreated rabbit.			Broth.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	$\frac{1}{2}$	5	1	1 small.	4+	1	$\frac{1}{2}$	5
2	$\frac{1}{2}$	5	2	1 medium sized.	4+	2	$\frac{1}{2}$	5
3	1 large.	5	3	" "	4+	3	$\frac{1}{2}$	5
4	$\frac{1}{2}$	5	4	" "	4+	4	$\frac{1}{2}$	0
5	1 large.	5+	5	$\frac{1}{2}$	5	5	$\frac{1}{2}$	5
6	$\frac{1}{2}$	5	6	2 large.	5	6	1 small.	5±
7	2 medium sized.	5+	7	2 medium sized.	4			

As shown in Table VI all the droplets showed growth except one of the broth, and growth occurred in all at approximately the normal rate. The cells in the immune blood formed chains characteristic of growth in homologous serum, but showed no lag as compared with the controls.

Similar series of cultures were made in the whole blood of the same rabbit taken $1\frac{1}{2}$ and 21 hours after the injection of serum. In neither series was there any evidence of inhibition of growth attributable to immune substances in the whole blood.

A series of test-tube experiments was then done with the whole blood, the serum, and the plasma of the immunized rabbit taken 3 days after the injection of the immune serum. Sowings of cells taken from an

actively growing Type I broth culture were made into the bottom of dry test-tubes. Into each tube only a single chain was sown, the chains varying in length from four to seven pairs each. Only sufficient broth to expel the chains from the pipette, amounting in each instance to a single small droplet, was discharged into the tubes. Three of the tubes so prepared received 1 cc. each of whole uncoagulated blood, not citrated; three received 2 cc. each of fresh serum; three, 2 cc. of fresh plasma; and three, 2 cc. of rabbit blood broth to serve as controls. The next day growth was evident in all except the three which received the whole blood. The growth appeared in the coagulated plasma in the form of single colonies in two of the tubes and as two colonies in the third. The growth in the serum and plasma was plated out and proved to be pneumococcus. The failure of growth in the whole blood might be attributed to phagocytosis, judging from the positive results obtained in hanging drops, but of this there is no definite proof.

On the same day the growth in plasma, in undiluted serum, and in whole blood from the same rabbit was compared with the growth in rabbit blood broth in hanging drops in much the same way as in the experiment described in Table VI. Growth failed in one of seven plasma droplets and in three of seven whole blood droplets. In the whole blood there was some evidence of phagocytosis in three of the droplets in which growth failed or was deficient. Growth occurred in all the serum droplets and in the broth controls. In all four sets when positive results were obtained, the amount of growth was approximately the same.

In summarizing all the experiments done with homologous serum, whole blood, or rabbit blood plasma *in vivo*, we find no measurable influence of the immune serum on the growth rate of pneumococcus.

Action of Homologous Serum in Vivo, in the Peritoneum of the Mouse.

In a series of experiments a study was made of the behavior of pneumococcus in the peritoneal cavity of normal and immunized mice. Mice were passively immunized by means of the injection of measured amounts of immune serum at varying periods prior to the injection of the bacteria. Sometimes the same mouse was used repeat-

edly for different experiments, so that it may have acquired some degree of active immunization by virtue of the repeated doses of bacteria. Cultures of different degrees of virulence and at different stages of growth were compared, and various sized doses of cocci were given. The object of the experiments was to ascertain whether the disappearance of the pneumococcus in the peritoneal cavity of the immunized mouse is due in any measure to the humoral activity of the peritoneal fluid. The protocols of several experiments will illustrate the method and results.

A mouse was given intraperitoneally 0.2 cc. of Type I antipneumococcic serum, and 1 hour later received 0.1 cc. of a rabbit blood broth culture of Type I pneumococcus, also intraperitoneally. The culture was the second transfer from the heart's blood of a mouse and had grown $15\frac{1}{2}$ hours at high room temperature. It was very cloudy and contained mostly discrete pairs or short chains. A test of viability was made at the time of inoculation by sowing single pairs of cells into hanging drops of broth. Twenty-six out of twenty-seven of these pairs grew, but the early growth was at only about half the normal rate. So practically all the cells inoculated were viable but not at the full height of growth.

At the same time that the immunized mouse was inoculated, a normal control received exactly the same dose of the same culture.

Samples of peritoneal fluid were taken from both mice by means of capillary pipettes 1 hour and 10 minutes, 4 hours, and 6 hours and 10 minutes after the inoculation of the bacteria. Additional samples were taken from the immunized mouse at about 8 hours (7 hours and 55 minutes) and at 28 hours after inoculation. In the control mouse numerous leucocytes and numerous cocci, mostly in separate pairs, were found at all periods. Phagocytosis, if present at all, was little marked. The mouse died during the night following the inoculation of pneumococci. In the immunized mouse many leucocytes and a few extracellular cocci, mostly in short chains, were found at the end of the 1 hour and 10 minute period. Some of these free chains were isolated into hanging drops of peritoneal fluid and grew at apparently the normal rate. Phagocytosis was marked, and the number of cocci found in phagocytes was apparently sufficient to account for the diminution of free cocci in the peritoneal fluid. At the end of the 4 hour

period and thereafter no extracellular bacteria could be found, although they were distinctly demonstrable in leucocytes at the end of 8 hours.

It was demonstrated that cocci contained within leucocytes of the immunized mouse were viable as late as 6 hours after inoculation and 2 hours after the period when extracellular cocci had apparently disappeared. This was proved by the following experiment.

Hanging drops made of the peritoneal fluid both undiluted and diluted in broth were allowed to incubate, and were then stained by means of a capillary pipette under microscopic control. These preparations were compared with similar preparations made from drops which were stained before incubation. In some leucocytes there was apparently no growth of cocci during incubation and the bacteria in some instances appeared to have degenerated. In others, small colonies had formed in and around the phagocyte. No evidence of bacteriolysis was found which could be ascribed to the effect of the homologous serum. A few free bacteria stained irregularly, but in no larger proportion than may be found in normal serum.

Hanging drop and plate cultures of peritoneal fluid were in all cases compared with films on slides subsequently stained by Gram's method and with Manson's methylene blue. The immunized mouse remained alive and well 6 days after the experiment.

In another experiment extracellular cocci taken from the peritoneal fluid of an immunized mouse were isolated in hanging drops containing a mixture of peritoneal fluid and broth. These cultures showed growth at fully the normal rate, five generations in $2\frac{3}{4}$ hours. In this case the mouse had received 0.2 cc. of homologous serum, followed by 0.05 cc. of a culture of Pneumococcus Type I, the first transfer from the heart's blood of a mouse. Tests in hanging drops had shown that practically all the bacteria inoculated were viable. This mouse had received no injection of bacteria or serum previous to the day of the experiment. The peritoneal fluid for the test was removed 35 minutes after injection of the bacteria. Phagocytosis was marked. After 1 hour and 25 minutes the fluid contained no free bacteria.

Growth Rate in Vivo in the Peritoneal Fluid of the Passively Immunized Mouse.

As noted in the previous experiments non-phagocytosed pneumococci isolated from the peritoneal cavity of the mouse grow in hanging drops at the normal rate. It was next proposed to ascertain whether growth takes place *in vivo* in the peritoneal fluid and to attempt to determine approximately the rate of growth, advantage being taken of the tendency of Pneumococci Type I to adhere in chains in homologous serum. These experiments were suggested by the observation that extracellular pneumococci found after 1 or 2 hours in the peritoneal cavity were usually in chains. One protocol will illustrate the method and results.

A mouse was given intraperitoneally 0.2 cc. of Type I serum. About $1\frac{1}{2}$ hours later it was given a dose of Type I culture. The inoculum was prepared as follows: A rabbit blood broth culture was incubated until it became well clouded and examination of hanging drops showed that most of the chains had broken up. A portion of the culture was then centrifuged at low speed for about 5 minutes, in order to throw down the chains. The supernatant fluid was nearly clear and hanging drop examination showed that no long chains remained. A series of cells was isolated in hanging drops and all proved viable and actively dividing. 0.4 cc. of the supernatant fluid was inoculated intraperitoneally. A sample of peritoneal fluid was taken in about 2 minutes, a second sample 60 minutes later, and further samples at 30 minute intervals. The samples were placed on slides with little spreading, dried, fixed in alcohol, and stained with Manson's methylene blue. The incidence of pneumococci in pairs or in chains of various lengths was noted in several fields taken at random. The results of the countings are given in Table VII.

Samples taken at 150 minutes and later showed nearly all the chains either in phagocytes or broken into irregular groups. Up to the fourth sample the chains were fairly intact, owing to the capsular material which held them together. A few chains in the fourth sample had begun to break up. Phagocytosis became marked at 90 minutes, bacteria became fewer, and an increasing proportion of the contents of the phagocytes consisted of longer chains. Few single pairs were found at the end of 60 minutes. The mouse recovered.

By comparing the incidence of chains in the different samples in Table VII it becomes evident that growth was going on at a rate of about one generation in 30 minutes. In the first sample most of the cocci were in single pairs, with the next greatest number in chains of two pairs. 60 minutes later the greatest number of cocci were in chains of 4 and 4 to 8 pairs. 30 minutes later the largest number of cocci were in chains of 8 and 8 to 16 pairs. This indicates that the rate of division in the peritoneal cavity approximates the usual rate observed in hanging drop cultures.

TABLE VII.

Length of Chains of Pneumococci in Samples of Fluid Recovered from the Mouse Peritoneum.

Sample No.	Length of time after inoculation.	No. of pairs of cocci per chain.									
		1	2	2–4*	4	4–8	8	8–16	16	16–32	32
	min.										
1	2	94	41		4						
2	60	1	2	3	31	10	5	1			
3	90					6	25	17	7	1	
4	120	†					3	7	23	11	9

* The headings 2–4, 4–8, 8–16, 16–32 refer to chains some members of which were dividing to form chains of a higher order.

† Some single pairs occur in clusters, probably from broken chains. Three 3 pair chains, four 6 pair, and six irregular chains are not included.

A parallel experiment with Type I pneumococcus was done on a mouse which had received during the 10 days previous to the experiment three doses approximating 0.05 to 0.1 cc. each of broth culture of pneumococcus together with homologous serum. These inoculations had been made in connection with other experiments and some active immunity may have been conferred on the mouse. This mouse was passively immunized on the day of the experiment and almost exactly the same procedure was followed as in the experiment described in Table VII. The results were similar except that in this case the chains held intact longer and could be counted at the end of 205 minutes, when chains of 64 to 128 could be found. About the same growth rate was observed as in the previous experiment. This mouse also recovered.

Similar experiments were also done with Pneumococcus Type II. This type proved less favorable, since the chains lost their identity earlier and tended to fuse into masses, owing to the more abundant formation of capsular material. In one experiment, however, three samples taken at about 30 minute intervals gave similar results to those of the Type I experiments. In this experiment only 0.2 cc. of supernatant culture fluid was given. Phagocytosis began in about 30 minutes and continued actively. However, this mouse died 4 days after inoculation with pneumococcus.

Apparently the growth of extracellular pneumococci in the peritoneal cavity of the immunized mouse continues until the chains or groups are overtaken by phagocytosis, and the growth of some portion, at least, of the cells continues at a geometrical rate. A few chains were noted in which a part of the members failed to take the stain deeply. This can hardly be considered as due to the action of the immune serum, since a similar phenomenon is sometimes observed in chains formed in undiluted normal blood or serum. At all events, the proportion of such degenerated cells in the mouse peritoneum is too small for us to ascribe much importance to this phenomenon as a factor in the disappearance of the bacteria.

Capsule formation was marked in the peritoneal cavity of the immunized mouse, but in Type I pneumococcus infection this apparently interfered little with phagocytosis. In Type II infection groups of bacteria formed thick gelatinous masses, which may have offered some mechanical hindrance to the phagocytes, but there is no proof in these experiments that these capsules were an important element in the protection of the bacteria.

DISCUSSION.

The studies which have been described, in which the single cell method of cultivating bacteria was employed, have not been successful in demonstrating that immune antipneumococcic serum owes any of its effect to any power of inhibiting the growth of pneumococci. While it is still possible that the serum may have such an effect, it has not been possible to demonstrate it by the method employed. That antipneumococcic serum has a high protective and curative

value has been demonstrated, but the mechanism of this action is still not entirely clear. The attempts to analyze its mode of action have so far only succeeded in showing that it has the power of causing marked agglutination of homologous organisms and of changing the organisms so that they undergo phagocytosis. The work of Bull (7, 8) shows that the agglutination of bacteria *in vivo* is probably a more important phenomenon in immunity and recovery than was formerly believed. The fact that the protective power of any lot of antipneumococcic serum, as tested *in vitro*, is not always proportional to its agglutinating power is opposed to the assumption that agglutination is of paramount significance. The exact importance of the property of facilitating phagocytosis has also not been thoroughly determined. The fact that the only real test of antipneumococcic serum is its action *in vivo*, in protecting the life of infected animals, seems to indicate that its effect depends, in part at least, on properties which are only manifested *in vivo* and the nature of which are not yet fully understood.

CONCLUSIONS.

1. Under the conditions stated pneumococci grow as readily in the serum of horses highly immunized to the homologous organism as they do in normal horse serum, and the rate of growth is not appreciably diminished.

2. This failure of immune serum to affect the growth rate is not altered when fresh rabbit blood, fresh human blood, or rabbit blister fluid is added in order to supply any hypothetical complement which might be lacking.

3. We have not been able to show that when immune horse serum is injected intravenously into rabbits or intraperitoneally into mice, it acquires the property of killing pneumococci or inhibiting their growth.

4. Experimental evidence has been obtained indicating that in the peritoneal cavity of the passively immunized mouse the growth of extracellular pneumococci continues at apparently the normal rate, until the bacteria are engulfed by phagocytes.

5. The immunizing and protective power of antipneumococcic serum probably depends, in part at least, on properties which are not

at present known. It has not been possible in the present study to demonstrate that one of these properties consists in delaying the growth of pneumococci.

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ANTIBLASTIC PHENOMENA IN ACTIVE ACQUIRED IMMUNITY AND IN NATURAL IMMUNITY TO PNEUMOCOCCUS.

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In the preceding paper (1) the results are reported of an investigation of the power of the serum of highly immunized horses to inhibit the growth rate of pneumococci. In that investigation, in which the so called single cell method of cultivating organisms was employed, it was impossible to demonstrate any growth-inhibiting action of the serum. This was true even when the serum was placed in the peritoneal cavity of the mouse and under conditions in which its full protective action was being manifested. Nevertheless, it seemed important to exclude the possibility that while no inhibiting action by serum can be demonstrated, the whole blood when freshly drawn from the actively immunized animal may have an effect of this kind. It seemed possible that the so called antiblastic immunity might play a part in active immunity, although the serum of the actively immunized animals might exert its effect in producing passive immunity in an entirely different manner. Consequently, a series of experiments was undertaken to determine whether or not blood or plasma fresh from the immune horse had any effect on the growth rate of pneumococcus. The same single cell method of study employed in the previous investigation was used in this work.

A virulent strain of Pneumococcus Type II was used in all the tests, and the blood was furnished by a horse which had received many injections of the same strain and of various atypical Type II strains as well. The degree of immunity was such that 0.2 cc. of serum protected mice against 10,000 lethal doses of the Type II strain employed in these experiments.

Tests with Whole Fresh Blood and Fresh Serum Diluted in Broth.

In the first series of experiments broth dilutions of whole blood and fresh serum were compared with fresh normal horse serum controls. Both hanging drop and test-tube series were done, the latter with sowings of chains of one to three pairs into quantities of medium varying from 4 to 10 cc. The whole blood was diluted 1:11 and the serum 1:5. In all the tests growth occurred in the dilutions of the immune blood as well as in the controls. The growth rate, as shown by growth in hanging drops, was practically the same in the immune blood and serum as in the controls.

Tests with Whole Citrated Blood.

The citrated blood was used on the day of collection, with normal horse serum as a control, both undiluted. The normal horse serum had been kept some months in the ice chest. The results after 2 hours incubation are given in Table I.

TABLE I.

Growth in Hanging Drops of Whole, Undiluted Citrated Blood of an Actively Immunized Horse Compared with That in Hanging Drops of Undiluted Normal Horse Serum.

Pneumococcus Type II.

Immune citrated blood.			Normal horse serum.		
Drop No.	Sowing. Pairs.	No. of generations formed after 2 hrs.	Drop No.	Sowing. Pairs.	No. of generations formed after 2 hrs.
1	2 small.	4	1	1 medium sized.	3+
2	2 "	4	2	2 small.	3+
3	1 medium sized.	4+	3	1 large.	4−
4	2 " "	4+	4	1 medium sized.	4−
			5	1 large.	4

There was therefore no evidence in these experiments that the whole fresh citrated blood from the immunized horse caused any delay in the growth rate of homologous pneumococci. Further experiments were made with fresh citrated blood in test-tubes, diluted and undiluted, but no definite evidence of inhibitory action could be obtained.

It was then determined to test the effect of whole undiluted blood transferred directly from the ear of the horse to sowings of one and two pairs of pneumococci previously isolated on the cover-glass, the method previously described being followed. Fresh normal horse blood was used as a control. After 3 hours incubation the greatest amount of growth in any droplet was two generations, and few showed that much. The amount of growth in the blood of the immunized horse and in the control was about the same.

Many hanging drop experiments were also done with the fresh undiluted coagulated plasma of both the normal and the immunized horse. In obtaining plasma for these experiments, blood contained in centrifuge tubes was placed on ice immediately after draining from the animal. The uncoagulated blood was centrifuged free from cells and kept cold until ready for use. The plasma coagulated soon after exposure to higher temperatures, but remained fluid long enough to allow mixing with organisms previously isolated in test-tubes or on the cover-glass. In every case without exception growth either failed or was limited to one generation after $2\frac{1}{2}$ hours of incubation. There was rarely, if ever, any trace of bacteriolysis, however, and the organisms sown could usually be found after incubation. Broth controls of the seeding material grew well. The effect of coagulated plasma was also tested by mixing it in amounts of 2 cc. in the bottom of test-tubes with minute droplets of broth containing six to eight pairs of pneumococci. Growth almost always occurred both in the tubes containing plasma from the immunized horse and in those containing plasma from the normal horse. There was some evidence that growth under these conditions tends to lag, however, but this effect was observed both in the plasma of the immune and in that of the normal horses. Similar experiments were done with the fresh undiluted serum of the same normal and immune horses. Here too it was found that in hanging drops a marked prolongation of generation time occurred which was evident both in the normal and in the immune serum.

These experiments indicate that under the conditions described whole fresh horse blood and coagulated plasma, and also serum, are not favorable media for the growth of pneumococci. They do not

indicate, however, that this fact is at all related to the phenomena of immunity, since it is exhibited in both immune and normal horse blood.

Natural Immunity.

While the previous experiments have not afforded any evidence that inhibition of growth of pneumococci by serum or body fluids plays any part in acquired immunity, it is possible that natural immunity against pneumococcus infection may depend to some extent upon this property of the serum. The inhibiting effect of whole horse blood upon the growth of pneumococci might give some support to this view. The relative susceptibility of the horse to pneumococcus infection is not accurately known, but it is not generally considered to be very high as compared with that of the mouse or rabbit, for instance. Certain experiments previously performed (1), while not planned with this point in mind, give strong evidence that whole rabbit blood, unlike horse blood, does not have an inhibiting effect upon the growth of pneumococci.

Heist, Solis-Cohen, and Solis-Cohen (2) have reported that by a special method which they have devised, they have demonstrated a bactericidal action in the blood of chickens and pigeons, animals which are known to be highly immune to pneumococcus infection. Strouse (3), who studied the question of natural immunity of the pigeon, was unable to demonstrate any bactericidal action of pigeon serum and presented evidence to show that this immunity was due to the normal high temperature of the pigeon which was inimical to the growth of pneumococci. Kyes (4) also found no evidence of pneumococcidal action of pigeon serum *in vitro*.

Heist, Solis-Cohen, and Solis-Cohen studied fresh whole pigeon blood. They used small glass pipettes, and sowings consisted of the small numbers of bacteria which adhered to the walls after the pipettes had been filled and emptied from broth cultures of pneumococci. The fresh blood is then brought directly into contact with these bacteria by sucking the blood into the tube. The tubes are sealed off, incubated, and the contents later blown on glass slides and stained. It is stated that by this method, with not only the undiluted broth cultures, but dilutions of the broth cultures as well for seeding, it is possible to ascertain whether bactericidal action has occurred and to what degree. They state that with this method they have demonstrated that pneumococci fail to multiply in pigeon blood. Chicken blood is also bactericidal but less so than pigeon blood, while normal rabbit blood and mouse blood have no such action. Immune rabbit

blood, however, has been shown in this way to have a specific bactericidal action, and they state that they have also demonstrated this action in the blood of recovered patients.

The method employed by these writers is, in our opinion, open to certain objections, and it seemed important to investigate this matter further, since it has a direct bearing on our problem.

In our experiments the hanging drop method with small sowings was employed. A minimum of broth was used in the isolation of the organisms, in some instances scarcely more than that of the volume of several erythrocytes. A record kept of the relative amount of broth employed showed that there was apparently no greater amount of

TABLE II.

Growth in Hanging Drops of Fresh Citrated Pigeon Blood Compared with That in Hanging Drops of Rabbit Blood.

Pneumococcus Type I.

Rabbit blood.			Pigeon blood.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	1 large.	3+	1	1 large.	2
2	1 "	3+	2	1 "	2—
3	1 "	3±	3	1 "	2—
4	1 "	3	4	1 "	2—
5	1 "	3+			

growth in the drops which were intentionally given a slightly larger amount of broth. After the isolations had been made, a relatively large drop of blood was added to each seeding in such a way that the blood was caused to engulf the minute droplet containing the isolated bacteria. By this method the behavior of an exactly known sowing could be followed under conditions in which any humoral factor might act, but in which there was a minimal chance of any phagocytosis occurring without being observed.

In two series of experiments fresh citrated undiluted pigeon blood was used. A protocol is given in Table II. A Type I pneumococcus culture, apparently somewhat past the maximum height of growth, was used for seeding. The time of incubation was 2 hours. Growth

occurred in all the droplets in both samples with a tendency to lag in both. The growth in the pigeon blood was about one generation behind that in the rabbit blood. The cocci tended to form chains in the pigeon blood and discrete elements in the rabbit blood.

A second experiment with virulent Type II culture gave essentially the same results.

In another experiment non-citrated blood taken from another pigeon was used. The organisms were isolated in a minimum of broth as before. Fresh non-citrated rabbit blood was used as a control. The animals were brought to the microscope and the blood was transferred by means of a capillary pipette to the droplets before coagulation had occurred. In one series the droplets were incubated for 5 hours in order to ascertain whether growth in the blood would continue beyond the first three or four generations. Actively dividing Type I cells taken from a broth culture were used for the sowings, which consisted of one pair or of a chain of two very small pairs per droplet. Of five droplets of rabbit blood all showed growth and gave approximately nine generations each. Of five droplets of pigeon blood, four showed growth, with approximately seven, six, two, and six generations respectively, a lagging of two to seven generations behind the growth in rabbit blood. The cocci in the rabbit blood tended to form groups of discrete elements, while those in the pigeon blood were mostly in chains.

Other experiments were made with the non-citrated fresh pigeon blood, and in these practically no difference appeared between the growth in rabbit and pigeon blood. A protocol of one of these is given in Table III. Sowings were taken from an actively dividing Type I culture, the same strain that was used in the previous experiments, and the time of incubation of the droplets was $2\frac{1}{2}$ hours.

Here the amount of growth was essentially the same in both blood samples, and in most droplets multiplication went on at the normal rate. In the pigeon blood there was a greater tendency to chain formation, and in the two droplets in which growth was deficient there was some evidence of phagocytosis.

In summarizing these experiments we may say that growth occurred about as often in the pigeon as in the rabbit blood, and the rate of growth in both was practically the same. By the method employed by

us, therefore, we were unable to demonstrate that pigeon blood has any bactericidal action or inhibiting effect on the growth of pneumococci. It is possible that the differences between our results and those of Heist, Solis-Cohen, and Solis-Cohen are due to the differences in the conditions of the experiment. However this may be, we have, by the technique employed, been unable to show any relation between natural immunity in the pigeon and inhibition of growth of pneumococci by the whole blood.

TABLE III.

Growth in Hanging Drops of Non-Citrated Pigeon Blood Compared with That in Hanging Drops of Rabbit Blood.

Pneumococcus Type I.

Rabbit blood.			Pigeon blood.		
Drop No.	Sowing. Pairs.	No. of generations formed.	Drop No.	Sowing. Pairs.	No. of generations formed.
1	½	5	1	½	4+ or 5
2	½	5	2	1½	2±
3	½	0	3	½	5
4	1 small.	5±	4	1 large.	5
5	½	4+	5	½	5
6	½	4+ or 5	6	1 small.	5
			7	1 "	3 or 4

SUMMARY AND CONCLUSIONS.

The paper gives the results of a series of experiments made with a special technique for the purpose of testing whether or not so called antiblastic phenomena or bactericidal phenomena play any part in immunity to pneumococcus. The technique employed is that devised by the writer and consists in the isolation of single bacteria and their growth in hanging drops where growth can be continuously observed. From these experiments it may be concluded that: (1) Whole fresh blood, coagulated plasma, or serum of the immunized horse added directly to pneumococci, has considerable inhibiting action on the growth of pneumococci. The inhibiting effect of the fresh blood, coagulated plasma, or serum of a normal horse, however, is as marked as that of a horse highly immunized to pneumococci of the same type.

This property, therefore, does not seem to be of importance in acquired immunity in the horse. No such property has been demonstrated in whole fresh rabbit blood. (2) It has been impossible to demonstrate that antiblastic phenomena play any part in natural immunity to pneumococcus, at least as far as the immunity of the pigeon is concerned. The whole fresh blood of the pigeon under the conditions employed exhibits no inhibiting action on the growth rate of pneumococci.

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BLOOD VISCOSITY.

I. Conditions Affecting the Viscosity of Blood after Withdrawal from the Body.

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Determinations of blood viscosity were taken up in this laboratory in connection with work on cyanosis and were found to be influenced by so many factors hitherto unconsidered that a more thorough investigation of the subject seemed warranted.

Viscosity may be defined as the internal resistance of the individual molecules of a liquid to passage over each other. It depends on the nature of the liquid and on the temperature. When a liquid flows through a capillary which is wet by it the movement of the layer of molecules next to the wall is nil and not dependent on the nature of the wall, while the movement in the center of the stream is the greatest.

According to Poiseuille (1), the volume of a liquid passing through a capillary tube is directly proportional to the 4th power of the diameter, the time, and the pressure, and inversely proportional to the length of the tube.

$$Q = \frac{d^4 pT}{l}$$

He showed that for each liquid there was a constant which was the reciprocal of the coefficient of viscosity for that liquid.

By the proportion

$$Q : \frac{d^4 pT}{l} :: k : \frac{1^4 . 1 . 1}{1}$$

the constant for the liquid is obtained

$$k = \frac{Ql}{d^4 pT}$$

and can be introduced into the equation thus

$$Q = k \frac{d^4 pT}{l}$$

by means of which the quantity Q can be determined for any capillary tube and pressure.

Since η, the coefficient of viscosity, is the reciprocal of k

$$\eta = \frac{d^4 pT}{Ql}$$

When a liquid flows through a vertical capillary under its own pressure,

$$p = h \cdot s$$

in which h is the height of the column and s the specific gravity of the liquid.

If we want merely the relative value of η we need only determine under similar conditions the outflow time of similar volumes.

$$\eta : \eta_1 :: \frac{Td^4 hs}{l} : \frac{T_1 d^4 hs_1}{l} = \frac{Ts}{T_1 s_1}$$

$$\eta = \eta_1 \frac{Ts}{T_1 s_1}$$

or since $\eta_1 = 1$

$$\eta = \frac{Ts}{T_1 s_1}$$

If the specific gravities of the liquids under investigation are not widely different

$$\eta = \frac{T}{T_1}$$

when T_1 is the outflow time of distilled water.

Method.

The specific gravity of most blood falls between 1,040 and 1,060, an average of 1,050. No attention has been paid in this work to the specific gravity of the blood, the viscosity simply representing the relation between the values of T for the blood under investigation and distilled water.

A viscosimeter of the type advocated by Determann (2) was made by a local glass-blower and, while differing from the orignal in having a capillary of slightly larger bore, gave satisfactory results as the value 0.073 minute for distilled water was repeatedly obtained.[1] The instrument requires only 0.2 cc. of blood for each determination and

[1] For a critical review of the different types of viscosimeters and the principles involved see Determann (8)

is provided with a water jacket and thermometer by means of which any desired temperature may easily be obtained. All determinations were made at 20°C. with a stop-watch registering to 0.001 minute, the instrument being cleaned and dried each time.

For work of the type planned it was impossible to use blood without some means of preventing coagulation. Defibrination, on account of the considerable changes it brings about (3), was not considered, and hirudine was not available. It has always been considered that the addition of oxalate increases the viscosity of the blood (3), but the work here reported shows that this change is apparently due either to insufficient mixing of the blood or to changes in its gas content. Small quantities of potassium oxalate were therefore used to prevent coagulation. Blood was withdrawn from an arm vein with a Record syringe and oxalated in various ways. At first the blood was immediately added to a small glass cup with a capacity of about 1 cc. containing a few grains of powdered oxalate. Later powdered oxalate was placed in the barrel of the syringe before drawing the blood, and finally the most satisfactory method was found to be that of wetting the inside of the syringe with a saturated solution of oxalate.

EXPERIMENTAL.

Effect of Oxalate on the Blood.

Experiment 1.—Blood was taken from an arm vein, put immediately into the small glass cup, the viscosimeter filled to the mark by suction from its opposite end, and the outflow time determined. Without removing the needle from the vein, more blood was taken into a Record syringe containing a little powdered oxalate. After considerable rotation to dissolve the oxalate, the viscosimeter was filled from the glass cup with this blood. The figures below show the results.

	Viscosity.
Unoxalated blood	7.15
Oxalated "	7.76

Experiment 2.—Blood was taken from an arm vein into a Record syringe wet with a 20 per cent solution of oxalate. The viscosimeter was immediately filled from the nozzle of the syringe and the outflow time determined. The remainder of the blood was put into a test-tube where it did not coagulate. Blood was taken as soon as possible from the same vein into another Record syringe wet with 0.9 per cent salt solution. The viscosimeter was immediately filled from the nozzle of the syringe and the viscosity determined.

	Viscosity.
Oxalated blood	7.02
Unoxalated "	7.02

Experiment 3.—Experiment 2 was repeated, but in filling the viscosimeter with the unoxalated blood a bubble of air entered the capillary. This had to be expelled and the instrument was refilled, with a consequent wait of a few moments. The delay was not sufficient to result in coagulation of the blood before the determination was made.

	Viscosity.
Oxalated blood	6.49
Unoxalated "	6.69

In Experiment 2 it will be noticed that there was no opportunity for change in the gas content of the blood and no chance for settling of the formed elements, as the viscosimeter was filled immediately from the nozzle. At the same time oxalate was present in sufficiently large amounts to prevent coagulation of the blood later in a test-tube. In Experiment 1 a few moments were spent in rotating the syringe to dissolve the oxalate. This gave opportunity for uneven distribution of the corpuscles and a large bubble of air due to the presence of dry oxalate in the syringe perhaps slightly changed the carbon dioxide content. These two factors resulted in a higher value for the oxalated blood. In Experiment 3 the viscosity of the unoxalated blood was greater, due evidently to settling of the corpuscles while the viscosimeter was emptied and refilled. It cannot be denied that when blood is oxalated in the manner described in Experiment 2 and the viscosimeter filled immediately from the nozzle of the syringe, there is not time for the blood and oxalate to mix thoroughly, and on this account the objection might be raised that in Experiment 2 the figure did not represent the viscosity of oxalated blood. It did not seem possible to overcome this feature in any way which would secure a uniform suspension of the red cells and a gas pressure the exact equivalent of that in the veins.

Effect of Different Quantities of Oxalate on the Same Blood.

Experiment 4.—15 cc. of blood were taken from an arm vein and 5 cc. put immediately into each of three separating funnels of 300 cc. capacity containing respectively 0.2, 0.4, and 0.6 gm. of dry powdered oxalate. Each funnel was im-

mediately stoppered, filled with cooled alveolar air,[2] and rotated for 1 minute. This operation was repeated three times or until no further increase in redness was observed. Each funnel was again carefully rotated for 1 minute in a horizontal position but with a constant slight alternate raising of each end, and blood was then drawn into a small glass cup for the viscosity determination by turning the stop-cock at the lower end.

	Viscosity.
Funnel 1 (0.2 gm. of oxalate)	7.26
" 2 (0.4 " " ")	7.26
" 3 (0.6 " " ")	7.26

It seems clear from this that, other conditions being the same, the blood viscosity is not affected by varying amounts of oxalate. With the evidence obtained here and in Experiment 2 it is probably justifiable to conclude that small quantities of potassium oxalate do not alter the viscosity at all.

Effect of Insufficient Mixing of the Blood.

A very uniform suspension of red cells is necessary if correct viscosity determinations are to be made. This is shown by Experiment 5.

Experiment 5.—20 cc. of blood were taken from an arm vein into a Record syringe wet with a saturated solution of oxalate, and the viscosity was determined almost immediately by filling the instrument directly from the nozzle of the syringe. At frequent intervals afterward the blood in the syringe was mixed by rotating the syringe so as to move about a bubble of air which had been allowed to remain in it and the viscosity determined by filling the instrument from the nozzle. After again mixing the remaining blood as thoroughly as possible in the syringe, one-half was placed in each of two separating funnels, which were immediately stoppered and rotated for 1 minute in the manner already described. Blood was then taken from each by the stop-cock and the viscosity determined.

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[2] In order to keep the carbon dioxide content of the blood about the same as that of the body, blood was often saturated with alveolar air which had been cooled to room temperature by passage through a vessel full of glass beads. As is shown in Paper II, this procedure sometimes actually increased the carbon dioxide content.

a. m.	Viscosity.
9.58 (immediately)	6.85
10.05	7.0
10.08	6.30
10.15	6.03
10.20	6.52
10.25	6.60
Funnel 1	6.90
" 2	6.96

The wide variation in viscosity in the first portion of the experiment was apparently entirely due to insufficient mixing of the blood as is well shown by the second portion. The viscosity after rotation in the separating funnel was slightly higher than the first and probably correct value, but this, as will be shown later, is to be expected from the slight loss of carbon dioxide.

Since the viscosity of the blood is affected by changes in its gas content, it was found convenient to secure a uniform suspension of the corpuscles by rotating the blood in a stoppered separating funnel, which at the same time insured an equilibrium between the gas content of the blood and the air in the funnel. Duplicate determinations were obtained without changing this equilibrium by opening the stopcock and drawing a small amount of blood into a 1 cc. glass cup. The time consumed in this manner before filling the viscosimeter did not seem to be sufficient to allow sedimentation of the blood.

Effect of the Amount of Blood.

The amount of blood in the funnel seemed to influence the results, as is shown by Experiment 6.

Experiment 6.—3 cc. of blood were placed in a separating funnel, stoppered, and then saturated with cooled alveolar air. The viscosity was then determined at intervals after carefully rotating the funnel each time for 1 minute.

	Viscosity.
Immediately	7.12
After 10 min.	7.4
" 30 "	7.68
" 60 "	6.99

When the results of Experiment 6 are compared with those in Experiment 5 it is evident that at least 5 cc. of blood must be used to insure accurate results.

Effect of Standing in Contact with the Air.

It has already been mentioned that the viscosity was found to be quickly affected by change in the gas content of the blood. Oxalated blood when left standing in contact with room air rapidly loses carbon dioxide and diminishes in bicarbonate content, as has been shown by Van Slyke and Cullen (4). If the blood is covered with a thin layer of paraffin oil this loss is greatly diminished in rate, or if the vessel containing the blood is tightly stoppered carbon dioxide is given off until an equilibrium is reached between the pressure of carbon dioxide above and that in the blood. Ewald (5) has noted that the viscosity of oxalated blood increased with the time after withdrawal. Experiments 7 to 9 show the effect on viscosity of allowing the blood to stand in an open vessel.

Experiment 7.—Blood was taken from an arm vein and placed immediately in a small glass cup containing a few grains of powdered oxalate. The viscosity was determined at once and at the end of 10, 30, and 60 minutes.

	Viscosity.
Immediately	6.16
After 10 min.	6.44
" 30 "	6.99
" 60 "	7.02

It is probable that stirring in the small glass cup did not give an absolutely uniform suspension of the red cells, yet the outflow time always increased in this and subsequent experiments, whereas if the change in viscosity had been due to unequal distribution of the corpuscles the results would have varied above and below the value obtained in the first determination.

Experiment 8.—10 cc. of blood were taken from an arm vein with a Record syringe containing a little powdered oxalate, placed in a separating funnel, saturated with cooled alveolar air, and stoppered. The viscosity was determined immediately and at intervals thereafter for an hour.

	Viscosity.
Immediately	5.48
After 10 min.	5.48
" 30 "	5.48
" 60 "	5.48

1 cc. of the same blood was placed in a small glass cup and the viscosity determined immediately and at intervals for an hour.

	Viscosity.
Immediately	5.23
After 10 min.	5.37
" 30 "	5.48
" 60 "	5.76

The difference in the initial values is attributable to an uneven distribution of the corpuscles before filling the small glass cup.

Experiment 9.—18 cc. of oxalated blood were divided into two portions, each of which was placed in a separating funnel, stoppered, and thoroughly rotated. The viscosity of blood from each funnel was taken. Funnel 1 was then left unstoppered for 4 hours and 35 minutes and the viscosity of this blood redetermined in duplicate.

	Viscosity.
Funnel 1	6.90
" 2	6.96
" 1 (after standing unstoppered for 4 hrs. and 35 min.)	8.25, 8.21

Experiments 7 to 9 show that the viscosity of oxalated blood constantly increases when it is allowed to stand in contact with the air, and this change can be prevented by tightly stoppering the container and rotating until carbon dioxide equilibrium is reached. The inference that the change in viscosity is dependent on the carbon dioxide content seems, therefore, justifiable.

Other observers (5) have shown that saturation of blood with carbon dioxide greatly increases the viscosity, and their findings have been confirmed in this laboratory. The influence of different partial pressures of carbon dioxide on blood viscosity is now under investigation and will be discussed in a later paper.

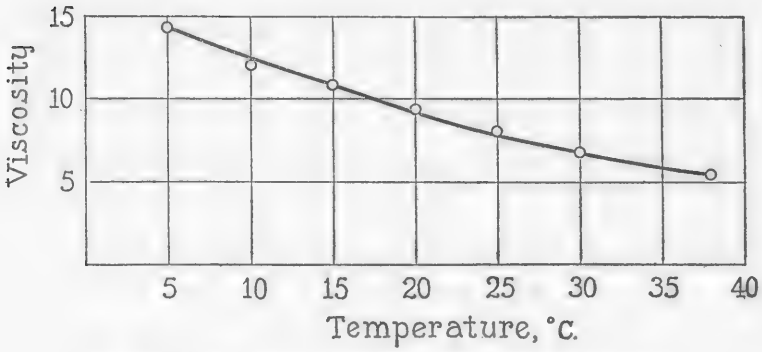
Effect of Temperature.

The blood viscosity varies greatly with temperature. It has been determined by some observers at body temperature (6) and by others at room temperature (7). Determann (8) reduced the value obtained at room temperature to 20°C. from a series of curves constructed by determining the viscosity of different bloods at different temperatures. Since all the observations in this work were made at 20°C., compari-

sons between different readings are reliable. In order, however, to study the effect of temperature on viscosity Experiment 10 was carried out.

Experiment 10.—10 cc. of blood were withdrawn from an arm vein into a Record syringe containing a little powdered oxalate, placed in a separating funnel, saturated with alveolar air, and stoppered. Blood was then withdrawn from the lower end into a small cup immediately before each determination, which was carried out in the usual manner after raising the water in the jacket to the required temperature.

Temperature. °C.	Viscosity.
5	14.41
10	12.47
15	10.96
20	9.40
25	8.03
30	6.85
38	5.48



TEXT-FIG. 1. The effect of temperature on the viscosity of blood.

When these values are plotted in the form of a curve as in Text-fig. 1, the line is almost straight. The viscosity of distilled water decreases directly with increase in temperature. With blood the viscosity is relatively slightly greater at body temperature than at lower temperature. This confirms the finding of Ewald (5).

CONCLUSIONS.

Small amounts of potassium oxalate probably have no effect on the viscosity of the blood and changes hitherto ascribed to it can be attributed either to variation in carbon dioxide content or to sedimentation of the red blood cells.

The viscosity of blood when exposed to the air increases rapidly. This change accompanies a loss of carbon dioxide and can be prevented by stoppering the container and agitating until the blood comes into carbon dioxide equilibrium with the air above it, when the viscosity remains constant.

It is essential in determining the viscosity of blood that the red cells should be uniformly suspended throughout the plasma. This can be accomplished by rotating 5 to 10 cc. of blood in a separating funnel for 1 minute.

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BLOOD VISCOSITY.

II. Effect of Increased Venous Pressure.

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Early in the study of blood viscosity the attempt was made to study the effect *in vivo* of increase of carbon dioxide on the blood viscosity by determining its coefficient before and after venous stasis induced by a loosely applied binder. The assumption was made that by raising the venous pressure for a time the passage of arterial blood through the capillaries would be delayed, the amount of carbon dioxide would be increased, and this would increase the viscosity, since it has been shown *in vitro* that saturating blood with carbon dioxide increases the internal resistance (1). It was thought that a concentration of red blood cells in the capillaries might accompany this increase in viscosity, for Cannon, Frazer, and Hooper (2) have shown that in shock the capillary count is higher than the venous.

Method.

The common method of taking blood from a finger, even when a spring lance was used, did not seem to offer the possibility of obtaining 0.2 cc. of blood rapidly enough to fill the viscosimeter without a considerable loss of carbon dioxide, and in addition it was found difficult to prevent small bubbles of air from entering the instrument. The attempt to use capillary blood for viscosity determinations was then given up and only venous blood used.

A few preliminary determinations of hemoglobin with the Palmer method (3) showed so rapid a deterioration of the standard that values obtained with it on different days were not comparable. This did not, however, affect the comparative value of determinations made on the same day, so that the 1 per cent standard was often used even though the absolute hemoglobin value was not obtained. Even

the 20 per cent solution of carbon monoxide hemoglobin as prepared in this laboratory sometimes faded rapidly in a few days. Thus a 20 per cent standard made on February 1 read 26 per cent lower when compared with a fresh one prepared on February 3. On the same day hemoglobin determinations made by the Palmer method on the same venous blood taken directly from the nozzle of the syringe with each of two 0.05 cc. pipettes, on some of the blood which had been poured into a test-tube and well agitated, and on a solution made by diluting 1 cc. of the blood to 100 cc. with 0.4 per cent ammonium hydroxide checked to within 1 per cent with the value found by the gasometric method of Van Slyke (4).

EXPERIMENTAL.

Effect of Increase in Venous Pressure on Venous and Finger Blood.

Experiment 1 shows the comparative concentration of venous and finger blood and the effect of increase in venous pressure on both.

Experiment 1.—Blood was taken with a Record syringe from an arm vein without any constriction, put into the small glass cup containing dry oxalate, and the viscosity determined in the usual manner. At the same time the finger was punctured for the red cell count and hemoglobin determination, the Palmer method being used for the latter. The blood pipettes had been carefully calibrated with mercury. A binder was then loosely applied to the arm, care being taken not to obstruct the flow of arterial blood into the arm. The determinations were then repeated after an interval of from 2 to 10 minutes. The results are shown in Table I.

TABLE I.

Venous blood		Finger blood.
Viscosity	6.80	
Red blood cells	6,490,000	6,265,000
Hemoglobin, *per cent*	133	130
Binder for 2 min.		
Viscosity	8.33	
Red blood cells	6,220,000	5,935,000
Hemoglobin, *per cent*	140	137

Experiment 2.—This experiment was similar in every respect to Experiment 1. The results are shown in Table II.

TABLE II.

	Venous blood.	Finger blood.
Viscosity	8.3	
Red blood cells	4,940,000	5,520,000
Hemoglobin, *per cent*	132	135
Binder for 2 min.		
Viscosity	9.40	
Red blood cells	5,800,000	5,980,000
Hemoglobin, *per cent*	141	143

Experiment 3.—This experiment was similar to Experiment 1. The results are shown in Table III.

TABLE III.

	Venous blood.	Finger blood.
Viscosity	8.69	
Red blood cells	6,450,000	6,250,000
Hemoglobin, *per cent*	143	143
Binder for 2 min.		
Viscosity	10.6	
Red blood cells	5,630,000	5,300,000
Hemoglobin, *per cent*	154	152

Except in Experiment 2 the venous count was always slightly higher than the finger count. That this is probably accidental is shown by the hemoglobin determinations which give no consistent difference between the finger and vein and check within a reasonable limit of error. It was concluded that in approximately normal individuals such as were used for this study there was no difference in the red cell concentration in the capillaries and veins that could be detected by these methods.

Effect of Increase in Venous Pressure on Hemoglobin.

After even so short a period as 2 minutes there was a very considerable rise in hemoglobin which was accompanied by a marked increase in viscosity. Experiment 4 confirms this increase in hemoglobin after venous stasis.

Experiment 4.—Blood was taken from an arm vein before and after application of a binder for 10 minutes and the oxygen-carrying power determined. From this the hemoglobin was calculated from Van Slyke's tables. The figures in Table IV show the results.

TABLE IV.

Hemoglobin, *per cent* (gasometric)	109.3
Binder for 10 min.	
Hemoglobin, *per cent* (gasometric)	119.1

Relation of Carbon Dioxide Content, Oxygen Unsaturation, and Oxygen-Carrying Power to Viscosity.

The attempt was made to determine whether this increase in viscosity was associated either with an increase in the carbon dioxide content or the oxygen unsaturation. Blood was taken before and after application of a binder and analyzed for total plasma carbon dioxide, plasma bicarbonate, or oxygen-carrying power and oxygen content.

Experiment 5.—Blood for the viscosity determination was taken from an arm vein with a Record syringe from which 1 cc. was immediately put into a small glass cup containing a small portion of powdered oxalate. The remainder was introduced by means of a glass tube and rubber connection into a centrifuge tube containing a little powdered oxalate and a small quantity of paraffin oil, and immediately centrifuged. The plasma was then pipetted off, the total carbon dioxide determined, and, after saturation with alveolar air, the plasma bicarbonate. Table V shows the results.

Experiment 6.—The procedure here was the same as in Experiment 5. Table VI shows the results.

Experiment 7.—This experiment differs only in the results, which are shown in Table VII.

Experiment 8.—This experiment is similar to the others. The results are shown in Table VIII.

TABLE V.

Viscosity	4.57
Total CO_2 in plasma, *vol. per cent*	57.6
Plasma bicarbonate, *vol. per cent*	65.3
Binder for 10 min.	
Viscosity	6.03
Total CO_2 in plasma, *vol. per cent*	52.8
Plasma bicarbonate, *vol. per cent*	60.3

TABLE VI.

Viscosity	6.56
Total CO_2 in plasma, *vol. per cent*	62.7
Plasma bicarbonate, *vol. per cent*	68.4
Binder for 10 min.	
Viscosity	8.63
Total CO_2 in plasma, *vol. per cent*	63.5
Plasma bicarbonate, *vol. per cent*	69.4

TABLE VII.

Viscosity	7.54
Total CO_2 in plasma, *vol. per cent*	59.5
Plasma bicarbonate, *vol. per cent*	60.5
Binder for 10 min.	
Viscosity	8.50
Total CO_2 in plasma, *vol. per cent*	65.3
Plasma bicarbonate, *vol. per cent*	69.1

TABLE VIII.

Viscosity	8.22
Total CO_2 in plasma, *vol. per cent*	63.4
Plasma bicarbonate, *vol. per cent*	65.5
Binder for 10 min.	
Viscosity	9.73
Total CO_2 in plasma, *vol. per cent*	55.8
Plasma bicarbonate, *vol. per cent*	59.6

In Experiments 5 to 8 there was a constant rather marked increase in viscosity following the rise in venous pressure. The plasma bicarbonate was constantly higher than the total carbon dioxide, but the value of neither followed consistently the viscosity, being higher in Experiments 6 and 7 but lower in Experiments 5 and 8 after increase in the venous pressure. It seemed evident from these results that the increase in viscosity following a rise in venous pressure could not be attributed to changes in the carbon dioxide content of the blood.

Experiment 9.—1 cc. of a 20 cc. sample of blood taken from an arm vein with a Record syringe was immediately placed in a small glass cup containing a very small portion of oxalate and the viscosity determined. The remainder of the specimen was passed through a glass tube and rubber connection into a test-tube containing a small amount of oxalate and a little paraffin oil and the oxygen content and oxygen-carrying power were determined. Table IX shows the results.

TABLE IX.

Viscosity	7.99
Venous O_2, *vol. per cent*	15.56
O_2-carrying power, *vol. per cent*	24.39
O_2 unsaturation, *vol. per cent*	8.83
Binder for 10 min.	
Viscosity	8.63
Venous O_2, *vol. per cent*	10.99
O_2-carrying power, *vol. per cent*	26.73
O_2 unsaturation, *vol. per cent*	15.74
Increase in viscosity, *per cent*	8.0
Decrease in venous O_2, *per cent*	29.4
Increase in O_2-carrying power, *per cent*	5.6
" in O_2 unsaturation, *per cent*	56.1

Experiment 10.—The procedure was the same as in Experiment 9. The results appear in Table X.

Experiment 11.—The procedure was similar to that in Experiment 9. Table XI shows the results.

There was a constant increase in the oxygen unsaturation values, though in Experiment 9 it was very large while in Experiment 11 it was comparatively small. The venous oxygen decreased markedly

in Experiment 9, very slightly in Experiment 10, and actually increased in Experiment 11. There was a large increase in the oxygen-carrying power in each case.

TABLE X.

Viscosity	6.64
Venous O_2, *vol. per cent*	13.71
O_2-carrying power, *vol. per cent*	24.90
O_2 unsaturation, *vol. per cent*	11.19
Binder for 10 min.	
Viscosity	7.12
Venous O_2, *vol. per cent*	13.25
O_2-carrying power, *vol. per cent*	30.37
O_2 unsaturation, *vol. per cent*	17.12
Increase in viscosity, *per cent*	7.3
Decrease in venous O_2, *per cent*	3.4
Increase in O_2-carrying power, *per cent*	21.8
" in O_2 unsaturation, *per cent*	29.2

TABLE XI.

Viscosity	6.6
Venous O_2, *vol. per cent*	11.05
O_2-carrying power, *vol. per cent*	24.13
O_2 unsaturation, *vol. per cent*	13.08
Binder for 10 min.	
Viscosity	7.74
Venous O_2, *vol. per cent*	11.95
O_2-carrying power, *vol. per cent*	26.88
O_2 unsaturation, *vol. per cent*	14.93
Increase in viscosity, *per cent*	17.3
" in venous O_2, *per cent*	8.1
" in O_2-carrying power, *per cent*	11.8
" in O_2 unsaturation, *per cent*	14.1

From the evidence obtained here it seemed impossible to associate the increase in viscosity with either the change in venous oxygen or the oxygen unsaturation. It ran more nearly parallel to the

rise in the oxygen-carrying power. This increase in the oxygen-carrying power could only be explained by a concentration of the blood in the capillaries; *i.e.*, by a loss of fluid from the blood to the tissues with consequent increase in the relative amount of hemoglobin. Such a concentration should be accompanied by an increase in the relative volume of the red blood cells, and experiments were therefore undertaken to determine whether this took place.

Experiment 12.—Blood was taken from an arm vein with a Record syringe and immediately placed in a small glass cup containing a very small amount of powdered oxalate. The 0.05 cc. pipette for determination of hemoglobin by the Palmer method and a capillary hematocrit tube were quickly filled from this cup. A binder was then loosely applied for 10 minutes and the determinations were repeated. The figures in Table XII show the results.

TABLE XII.

Hemoglobin, *per cent*	126.8
Hematocrit	41
Binder for 10 min.	
Hemoglobin, *per cent*	140.7
Hematocrit	54

Experiment 13.—Blood was taken from an arm vein and immediately placed in a test-tube containing a little powdered oxalate. A hematocrit tube was then filled and the remaining blood set aside for determination of the hemoglobin by the gasometric method. A binder was loosely applied for 10 minutes and the procedure repeated. The figures in Table XIII show the results.

TABLE XIII.

Hemoglobin, *per cent*	136.6
Hematocrit	47
Binder for 10 min.	
Hemoglobin, *per cent*	141.6
Hematocrit	50

These experiments showed that the increase in hemoglobin after rise in venous pressure was accompanied by a similar increase in the

relative volume of the red blood cells. In order to show *in vitro* the effect of an increase in the relative volume of the red cells on blood viscosity Experiment 14 was carried out.

Experiment 14.—16 cc. of blood were taken from an arm vein with a Record syringe containing a little powdered oxalate, 8 cc. being placed into each of two tubes and a few drops of paraffin oil added. After allowing the corpuscles to settle 0.8 cc. of plasma was removed from one of the tubes and both were then thoroughly stirred under oil by up and down movement of a stirring rod flattened at the lower end to nearly the inside diameter of the tube. 1 cc. of whole blood was removed from each tube and the viscosity determined.

	Viscosity.
Whole blood	9.59
Blood minus 10 per cent of volume in plasma	12.33

This experiment demonstrated that an increase in the relative volume of the red blood cells caused an increase in the viscosity of the whole blood.

It was of further interest to discover whether the fluid lost to the tissues was plasma or whether the plasma itself became more concentrated by loss of fluid and therefore more viscous. Experiment 15 was undertaken to determine this point.

Experiment 15.—15 cc. of blood were taken from an arm vein with a Record syringe containing a little dry powdered oxalate, and put into a centrifuge tube. A specimen was removed for determination of the oxygen-carrying power, a few drops of paraffin oil were added, and the remainder was centrifuged at high speed so that the plasma could be pipetted off. The total nitrogen of 1 cc. of plasma and the plasma viscosity were determined. A binder was loosely applied to the arm for 10 minutes and the procedure repeated. The figures in Table XIV show the results.

TABLE XIV.

O_2-carrying power, *vol. per cent*	24.3
Viscosity of plasma	1.89
Total nitrogen in 1 cc. of plasma, *gm.*	0.0105
Binder for 10 min.	
O_2-carrying power, *vol. per cent*	27.9
Viscosity of plasma	2.30
Total nitrogen in 1 cc. of plasma, *gm.*	0.0126

This experiment showed that a rise in venous pressure was accompanied by an increase in viscosity and total nitrogen of the blood plasma, and therefore by a concentration of the plasma. An increase in the viscosity of the plasma is therefore a factor in the increase in the viscosity of the whole blood after a rise in venous pressure.

CONCLUSIONS.

A rise in venous pressure caused by application of a loose binder to the arm results in a marked increase in the viscosity of the whole blood which is primarily due to a concentration of the blood in the capillaries. This concentration is shown by an increase in the viscosity and total nitrogen of the plasma, an increase in the relative volume of the red blood cells, and an increase in the relative percentage of hemoglobin.

Change in the viscosity of whole blood following venous stasis apparently bears no demonstrable relation to the carbon dioxide or oxygen content.

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THE EFFECT OF HYPOTONIC AND HYPERTONIC SOLUTIONS ON FIBROBLASTS OF THE EMBRYONIC CHICK HEART IN VITRO.

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In the literature on tissue culture are reported, more or less completely, experiments testing the effect of hypotonic and hypertonic solutions upon growth of cells and upon individual cell structures. It was with a view, therefore, of studying more fully the effect of solutions of different concentrations that the present work was undertaken. The experiments were performed in April, May, June, and July, when the weather was at summer heat and there was consequently little danger of chilling the delicate new growths, which are susceptible to any sudden change of temperature. This point is important, because several investigators have stated definitely that growth is much better in the spring and summer than in the fall and winter.

Carrel and Burrows were the first to try the effect of dilution and concentration of the medium upon tissue cultures, using the spleen of adult chicks and of 14 and 16 day chick embryos. Distilled water was added to the plasma to make it hypotonic, and sodium chloride to make it hypertonic. They found that growth in hypertonic plasma was always less than normal, while growth in the hypotonic solution (three volumes of plasma to two volumes of distilled water) was greatly accelerated. In fact, this medium gave the greatest amount of growth, much more than that obtained in normal plasma, a medium which these writers admit is not the best for this purpose.

Ruth found that by adding distilled water to plasma in which epithelial cells of frog skin were planted the growth was greatly increased.

Later, Lambert tried diluting the plasma with serum. He got the most extensive migration in plasma diluted with two parts of serum. Dilution with serum, Ringer's solution, and water gave about the same results. He therefore

concluded that "the most active migration took place in the plasma diluted with the largest quantity of fluid, and not in the medium made hypotonic by the addition of distilled water." This agrees with the work of Burrows (1913), who used spleen and diluted the plasma with serum. Both these observers attribute the increased migration to a diminution in the amount of fibrin in the clots; this makes a coarser meshwork which offers less resistance to the progressive movement of the cells. Lambert also finds that diluting the plasma affects differently the various types of cells. Actively motile cells, such as those of bone marrow and spleen, show increased migration; cells with limited power of locomotion, like those of intestinal epithelium, are not affected by the dilution of the medium; while cells of moderate motility, such as connective tissue cells, are only slightly influenced by it. Lambert concludes that this dilution of the medium has likewise no effect upon cell division.

Ebeling used hypotonic, hypertonic, and diluted media prepared by adding Ringer's solution to normal chick plasma and embryonic chick extract. Each of these media at first stimulated cell proliferation, but after a few days this decreased and ended in death unless measures were taken to revive the cultures.

Sundwall also grew tissues in hypotonic solutions, using plasma diluted with distilled water. He found the growth to be more prolific, but the difference between this and growth in plasma was not so marked as that obtained by Carrel and Burrows.

Cash used hypotonic salt solution on 3 day chick cultures and found that at once numerous vesicles appear, change shape characteristically for a short time, and then flow back into the cells. He states that it is difficult to produce these changes in younger cells; *i.e.*, in growths 1 to 2 days old.

Goldschmidt found that the direction of growth of the sex cells of the moth (*Samia cecropia* L.) was affected by hypertonic and hypotonic solutions. In the former the cells grew out against the follicle wall. The extent of outgrowth depended upon two factors—the age of the cell and the hypertonicity of the medium. The whole process was reversible without change of medium, as were also the slight changes produced by hypotonic media. Goldschmidt therefore concludes that the follicle membrane has active control of osmotic conditions inside the follicle.

Lewis and Lewis (1914-15) note a definite effect of hypotonic and hypertonic solutions upon the mitochondria; in the first they swell, while in the second they shrink. Cowdry states that very dilute solutions of formaldehyde cause swelling of the mitochondria, while a concentrated solution results in shrinkage.

Material.

For these experiments hearts of 6, 7, 8, and 9 day chick embryos were used. The medium was a modification of Locke's, known as Locke-Lewis solution. The Locke formula was as follows:

	gm.
Sodium chloride	0.9
Calcium chloride	0.024
Potassium chloride	0.042
Sodium bicarbonate	0.02

This was modified, according to Lewis' method (1915), by the addition of chick bouillon (15 cc. of bouillon and 0.25 gm. of dextrose to 85 cc. of Locke's solution), sterilized in clean test-tubes in an Arnold sterilizer, and kept in a moist chamber to avoid evaporation. In order to make it hypertonic the solution was boiled down one-fourth, one-half, etc., as required, and the amount of sodium chloride present was calculated. The hypotonic solutions were made by diluting the Locke-Lewis solution with distilled water, and in these also the amount of sodium chloride was determined. Since chick bouillon contains practically the same amount of sodium chloride as the Locke solution, the calculation of this content in the Locke-Lewis solution was made upon the same basis. It will be noted that only the amount of sodium chloride has been considered, although the other salts were present in definite amounts and always in the same proportion.

Method.

All the instruments and glassware used in the experiments were sterilized; the instruments were sterilized in a flame continually during the preparation of the cultures, so that infection of the cultures was rare. The solution was warmed and poured into small Petri dishes. The heart was aseptically removed from the chick embryo in Locke-Lewis solution and transferred to another Petri dish containing about 10 cc. of the medium. Here it was cut into small pieces and transferred by means of a pipette to a cover-glass, which was immediately inverted over a hard vaseline ring on a depression slide. The chance of evaporation by this method of the Lewises is very small, as the tissue can be placed on the cover-glass and sealed in a few seconds. When the tissue was to be planted in hypertonic or hypotonic solution the heart was placed in the liquid immediately after its removal from the embryo; the tissue was therefore never in contact with other solutions that might possibly dilute the medium.

The cultures were incubated in a constant temperature box at 39°C. Many of the slides were treated with neutral red which stains certain granules and vacuoles (Lewis, 1919). Janus black No. 2 was added when the mitochondria were to be studied, but only when early results were obtained from the experiment, as the cells do not live much more than an hour after being stained with it.

Normal Growth of Heart Tissue.

The normal growth of heart tissue is characteristic. Two types of connective tissue cells appear—fibroblasts and mesothelial cells. The fibroblasts (Fig. 1, *a*) are of more frequent occurrence and show two methods of growth: (1) migratory (Fig. 1), where the cells are scattered along the transplant and out into the medium; and (2) reticular, where the cells form a close reticular network. The latter is usually the more vigorous growth. These cells have long, branching processes, often so thin that it is impossible to follow them in their farthest wanderings and anastomoses with other cells. Hence, in most of the drawings of cells these processes are omitted, as they could not be seen with the camera lucida.

The mesothelial cells (Fig. 1, *b*) are of less frequent occurrence. They always appear in membrane formation (Lewis and Lewis, 1912), sometimes as small inclusions among the fibroblasts, and again as large growths completely surrounding the transplant. They are much larger and hardier, and withstood the action of the hypotonic and hypertonic solutions much better than the sensitive fibroblasts, with their fine, branching processes, which usually reacted at once to the new medium. For that reason, and also because they were the more common form, the fibroblasts were always used in the experiments.

As controls to the other experiments 282 cultures were planted in normal Locke-Lewis solution, and of these 188 (66.6 per cent) grew. The length of life in this medium varied. The oldest growth was 19 days old, and had been made from a 7 day chick embryo. It lived to this age without any change of the medium; in fact it was sealed with a vaseline ring during the entire time. The cells contained very few fat globules and towards the end of the experiment they withdrew their processes, only blunt ends remaining. They then accumulated in groups of five or six all over the slide.

Experiments with Hypotonic Solutions.

For these experiments three different solutions were employed: (1) three volumes of Locke-Lewis solution to two volumes of distilled water; this had a sodium chloride content of 0.540 per cent; (2) one volume of Locke-Lewis solution to one of distilled water; sodium chloride content 0.45 per cent; and (3) one volume of Locke-Lewis solution to three volumes of distilled water; sodium chloride content 0.225 per cent.

The first solution, which was the proportion used by Carrel and Burrows in their work on the spleen, was employed in two experiments with hearts of chick embryos 8 and 9 days old respectively. Out of twenty-three cultures seven gave reticular growth, thirteen migration, and three showed no signs of growth. In one experiment all were dead on the 4th day, while in the other some of the cultures lived for 7 days. Migration of cells was at first rapid as compared with the controls, but the latter lived a little longer. The percentage of growths in this medium is high (86.9), but only two experiments were performed. On these 2 days the percentage of growth of cultures in normal Locke-Lewis solution was 81.8—only slightly less. The number of reticular growths in the two media was practically the same—35 per cent in hypotonic and 30 per cent in Locke-Lewis solution, although in the former the extent of growth was greater.

Burrows (1913) and Lambert attribute the increased migration to a lessening of fibrin in the diluted plasma and consequent decrease in the resistance offered to migration. Here, however, this was not the case, as there was no fibrin in any of the cultures grown in the Locke-Lewis solution. The real cause seems to me to be the search for food which acts as a stimulus, as will be seen later. Burrows (1913) has also observed that tissues planted in plasma diluted with water suffer early death. This he thinks is due to the hypotonicity. He says: "They die apparently in the plasma diluted with salt solutions from an early exhaustion of food materials." This I believe is true, although when they die after being treated with (not grown in) hypotonic solution, it is possible that the cells have taken up too much water, which of itself is known to act as a poison,

causing death. When the individual cells were examined, a few were found to be long and fusiform and often small, though many were in a vigorous condition. When neutral red and Janus black No. 2 were added to the drop of culture fluid, both stains being made up in the hypotonic fluid used for the experiment, the neutral red granules were found to be arranged around the centriole near the nucleus, just as in the cells grown in Locke-Lewis solution. The mitochondria were long, sometimes branching, and often formed a network. Sometimes they extended down the processes to a considerable length. All these forms are as one usually finds them in normal growths.

The most striking point about the growths in this solution, and indeed in all hypotonic solutions, was that the cells nearest the explant died first (Fig. 2). This is exactly the reverse of the condition prevailing in all Locke-Lewis cultures and in the hypertonic growths, where the cells at the outer edge of the growth die first, until finally only those next to the transplanted tissue are left. These frequently live on for days after the main part of the growth has died. In the hypertonic solutions the migration is slower. There is a greater amount of food material (salts, etc., from the Locke solution and chicken bouillon) in the medium, so that the cells do not need to migrate so rapidly and consequently do not give off so much in the form of waste products. Hence the medium is not altered so rapidly (Burrows, 1913) and migration is retarded. In short, all depends upon the amount of food in the medium. When this is small, as in hypotonic solutions, the cells must migrate more rapidly to obtain food. They then give off more waste products which alter the medium, thus further stimulating movement. In a short time the accumulation of waste products becomes so great and the supply of food near the explant so small that the cells in that vicinity die. The same phenomenon was observed in *Amœba limax* cultures grown on agar (Hogue). The amebæ nearest the old point of inoculation on the Petri dish die before those which have wandered out over the dish, where they are free from waste products and can get more food and oxygen.

In the second hypotonic solution, made with one part of Locke-Lewis solution to one part of distilled water, 90 out of 202 explants grew (44.5 per cent). The migration was not so rapid as in the former

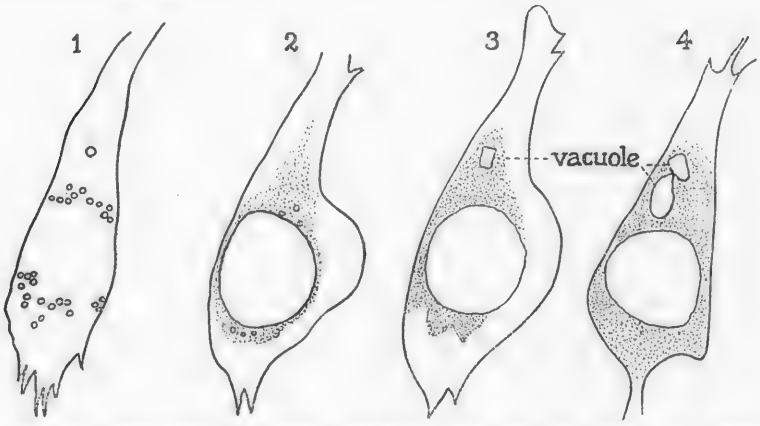
medium (0.54 per cent sodium chloride), and consequently the cells survived longer. On one slide they lived for 12 days without a change of the solution. As a rule, the cells appeared normal, with large growths in both reticular and migratory formation. The individual cells were normal in shape and the neutral red granules were usually the same as those in tissue grown in Locke-Lewis solution. Even the mitochondria presented a normal appearance. The cells seemed to have completely adapted themselves to their hypotonic environment. In this medium, as in the former one, the first cells to die were those next to the tissue explant.

The third hypotonic solution (one part of Locke-Lewis solution to three parts of distilled water, with a sodium chloride content of 0.225 per cent) gave no growth whatever after 48 hours of incubation. One drop of Locke-Lewis solution was added to some of the slides and the tissue again incubated in the hope of inducing migration, but the cells appeared to be dead.

Hypotonic Plus Hypotonic Solution.—The fact that excellent growths had been obtained on some slides of the Locke-Lewis 0.45 per cent sodium chloride medium while there was none on others planted the same day, in the same medium, and from the same heart, suggested the possibility that the particular explants that grew so well may have contained sufficient plasma of their own to change the medium. Accordingly, to the 24 and 48 hour growths in this medium was added some of the same fresh solution. On many slides the cells were all dead on the following day. On others the cells at the edge of the growth, which were very flat and thin with a large surface exposed, had been killed by the new hypotonic solution, while others had migrated out over them and were still in good condition (Fig. 3), showing that they retained the power of adapting themselves to the medium. It is not possible to determine here whether the explant had taken sufficient plasma with it to modify the medium, or whether the resistance of the individual cells was greater on some slides than on others. This individual difference in the cells is a factor always to be considered. It makes experimenting with tissue cultures a difficult problem, and one must perform a large number of experiments in order to draw any trustworthy conclusions.

Effect of Hypotonic Solutions on Normal Growth.

Hypotonic Solution, 0.225 Per Cent Sodium Chloride.—When a normal growth of 48 hours was treated with hypotonic Locke-Lewis solution with 0.225 per cent sodium chloride content, it was killed almost at once. The cells could therefore be stained with Janus black No. 2 without danger of interfering with the results of the experiment. After the growths had been stained with neutral red and Janus black, drawings were made of certain cells and the hypotonic solution was added.



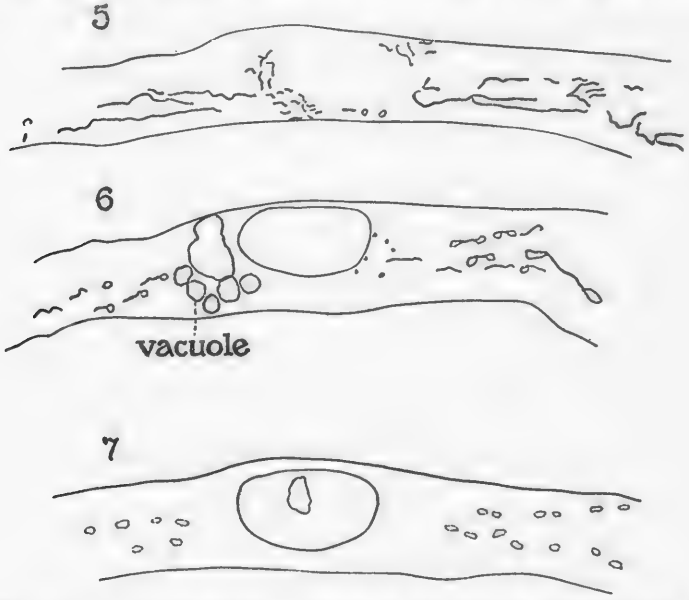
All the text-figures are camera lucida drawings made from living cells which have been stained with neutral red and often also with Janus black No. 2. The neutral red granules are represented as small circles, the mitochondria as solid lines or solid rods.

TEXT-FIG. 1. A normal fibroblast from a 48 hour growth explanted from a 9 day chick embryo heart and stained to show the neutral red granules.

TEXT-FIGS. 2 to 4. The same cell after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). Text-fig. 2 shows the cytoplasm much enlarged by the intake of water and divided into a granular and a clear area. The nucleus is swollen and has a distinct nuclear wall. A few neutral red granules are still visible. In Text-figs. 3 and 4 vacuoles are appearing. In Text-fig. 4 the cell is becoming smaller. Death changes have set in.

Many of the cells immediately took up a large amount of water. Text-fig. 1 shows a cell stained with neutral red, in which the gran-

ules are grouped together around the centriole. Text-fig. 2 represents the same cell 8 minutes after treatment with the Locke-Lewis sodium chloride 0.225 per cent solution. Here we have two kinds of cytoplasm, a granular part around the much swollen nucleus, and a clear outer area which has taken up water in the process of equalizing the osmotic pressure within the cell incident to the new environ-



TEXT-FIG. 5. Normal fibroblast from a 48 hour growth explanted from a 9 day chick embryo heart.

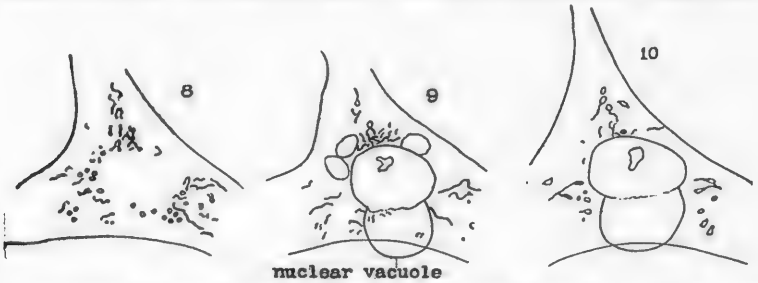
TEXT-FIG. 6. The same cell 5 minutes after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). The mitochondria are forming vesicles at their ends. Vacuoles are appearing in the cytoplasm.

TEXT-FIG. 7. The same cell 55 minutes after treatment. Only faintly staining vesicles remain where the mitochondria were.

ment. The processes have become more distinct as they, too, are full of the absorbed water. In Text-fig. 3 the neutral red color has disappeared from the granules and the nuclear wall is very distinct—both indications of cell death. In Text-figs. 3 and 4 vacuoles are appearing. Text-fig. 4 was drawn 4 hours, 25 minutes after the cell

was treated. Again the cell is small and most of the clear cytoplasm has been filled up with fine granules. This slide was later stained with neutral red, but the new vacuoles did not take up the stain, having undergone some death changes.

When the reaction of the cell to the hypotonic solution was not too sudden the effect on the mitochondria could be followed. Text-fig. 5 shows a cell with long branching mitochondria, drawn at 3.17 p.m. It was treated with hypotonic Locke-Lewis 0.225 per cent sodium chloride solution at 3.20 p.m.; by 3.25 p.m. (Text-fig. 6) no neutral red granules could be seen, the nucleus had a distinct membrane, vacuoles were appearing, and the mitochondria were beginning



TEXT-FIG. 8. Normal fibroblast from a 9 day chick embryo heart; 48 hours growth.

TEXT-FIGS. 9 and 10. The same cell 30 and 50 minutes after treatment with Locke-Lewis solution (0.225 per cent sodium chloride content). All the neutral red granules have disappeared. The mitochondria are forming vesicles. Vacuoles have appeared in the cytoplasm and a nuclear vacuole has formed.

to degenerate. In some, either one extremity or both seemed to swell (Text-fig. 6). Gradually the connection between these two small vesicles became fainter, then disappeared, and finally there remained only a faintly staining pale blue vesicle of irregular outline where a large mitochondrium had been (Text-fig. 7). The small mitochondria had disappeared entirely.

This process is also seen in Text-fig. 8 (normal), and in Text-figs. 9 and 10, drawn respectively 30 and 50 minutes after treatment.

Not all the mitochondria in a cell form these vesicles while undergoing degeneration, nor do those which do form them appear to bear any relation to the nucleus. Some are near it, some are farther away

near the cytoplasmic processes. In no case did I observe a swelling of the mitochondria as Lewis and Lewis (1914-15) and Cowdry claim occurs in hypotonic solutions. In every experiment the neutral red color disappeared from the granules almost as soon as the hypotonic solution was added. The mitochondria are much slower in disappearing.

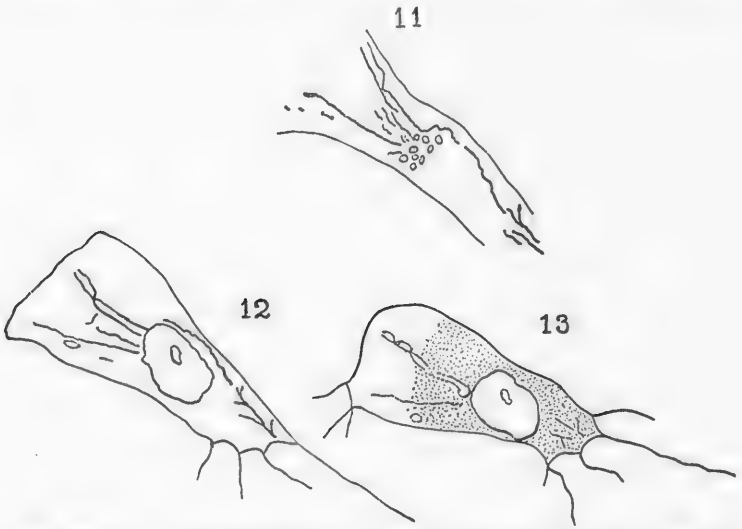
Hypotonic Solution, 0.3 Per Cent Sodium Chloride.—When the normal cells were treated with hypotonic Locke-Lewis solution with 0.3 per cent sodium chloride content, practically the same results as those recorded above were obtained. Certain mitochondria were followed and most of them simply faded from view. A few first formed a small vesicle at one end.

Hypotonic Solution, 0.45 Per Cent Sodium Chloride.—Hypotonic Locke-Lewis solution with 0.45 per cent sodium chloride content was tried on normal 48 hour growths. One cell (Text-fig. 11) with large, characteristic mitochondria was followed closely for 6 hours. Two large mitochondria in the anterior end were crossed; near them was a long, slender mitochondrium extending almost to the nucleus, and in the posterior end were some shorter ones with characteristic shapes. When treated with hypotonic solution the cell reacted at once. The color in the neutral red granules soon disappeared and the nuclear wall became distinct and irregular in outline. The mitochondria did not change shape at first but gradually became fainter (Text-fig. 12). The two peripheral ends of the long crossed mitochondria seemed to come together to form a ring (Text-fig. 13). About half way down the mitochondria there was a break and the parts nearer the nucleus became granular. About 1 p.m. it was noticed that these granular parts of the mitochondria seemed to be connected with a small clear vesicle near the nucleus, It looked as though they were forming a tubule which opened into the vesicle.

During the next 3 hours there was little change in the mitochondria, except that they grew more slender and faint. The cytoplasm around them became more granular, so that it was difficult to follow them. At 4.15 p.m. they were finely granular; this condition persisted for 3 days, when the slide was destroyed. The long mitochondrium to the right of those which were crossed did not appear so granular. It seemed, like the distal ends of those which were crossed,

to become more slender as though some substance were dissolving away from it. I could not observe, however, that any of the mitochondria became shorter.

In some of the cells treated with hypotonic Locke-Lewis solution large, clear vacuoles forming around the nucleus were frequently observed (Text-figs. 9 and 10). Others would form in the cytoplasm,



TEXT-FIG. 11. Normal fibroblast from a 9 day chick embryo heart, 48 hours growth, showing characteristic mitochondria and neutral red granules grouped around the centriole area.

TEXT-FIG. 12. The same cell 2 hours later. The neutral red granules have disappeared. The nuclear wall is distinct and the mitochondria are becoming fainter.

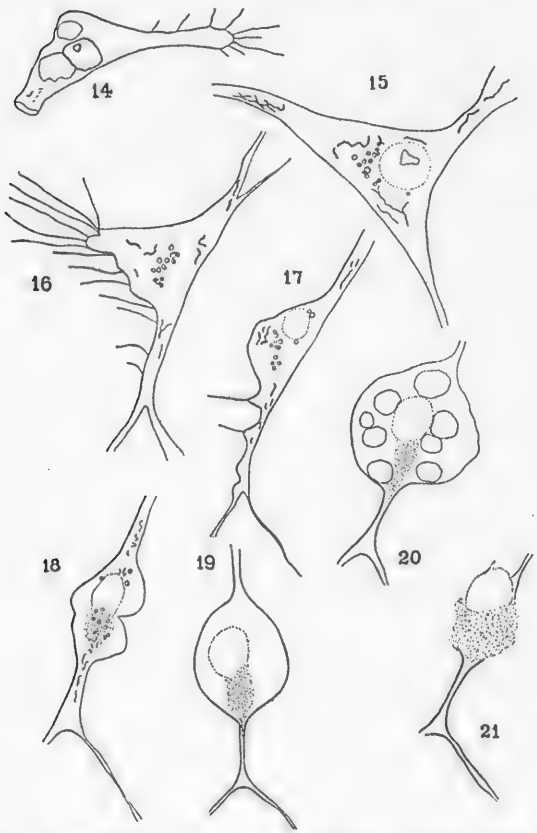
TEXT-FIG. 13. The same cell 2 hours later. The cytoplasm is granular. The two long crossed mitochondria are broken into pieces, some of which are granular. The other mitochondria are fainter or have disappeared entirely.

but these did not have the regular round or oval outlines of the nuclear vacuoles where the surface tension seemed to be very great. The nucleus, which was usually swollen in these cases, had taken up more water than it could hold and had broken out to form a vacuole. The nuclear wall near the vacuole was irregular in outline.

As already stated, the cells frequently become swollen with the intake of water as soon as they have been treated with the hypotonic solution. They remain in this condition for an hour or so, until the cell has begun to undergo the changes following death, when they show shrinkage. At times all the processes at one end are withdrawn and the end of the cell rolls back on the cell itself, as though from sudden contraction (Text-fig. 14).

A few experiments were tried with tissues which had failed to grow in Locke-Lewis 0.45 per cent sodium chloride solution. To each was added a drop of normal Locke-Lewis solution and the slides were again incubated, but no migration of cells ever appeared. A similar experiment was tried with tissue that would not grow in normal Locke-Lewis solution. Distilled water was added to the slides but the tissues did not respond, even after being in the incubator 48 hours. They appeared to have lost the power to migrate. They were not dead, for the pieces of heart frequently continued to contract, although cell migration could not be induced.

It was found that when hypotonic Locke-Lewis 0.45 per cent sodium chloride solution was added to normal growths it did not always kill the cells. A series of general experiments with this solution was tried with 24 and 48 hour growths. After thoroughly washing the cultures with the solution they were put in the constant temperature chamber at 39°C. and observed from hour to hour in a warm box. In a short time they took on a very different appearance from the normal. The cells were spider-like, their form completely changing from the flat, triangular, and fusiform shape, with long, fine processes almost invisible at the ends. Now the processes had a definite outline and seemed to anastomose, and the cell bodies had thickened into round or oval forms. Sometimes only the cells at one side of the explant would be thus affected, at other times those at one end, and again, all the cells. After 24 hours some of these cells would be dead, others would have recovered, and growth and migration would be going on beyond the dead cells. In one series of experiments pieces from the same heart were planted in hypotonic Locke-Lewis 0.45 per cent sodium chloride solution and in normal Locke-Lewis solution. Growth took place in both. When the controls were 24 and 48 hours old they were treated with hypotonic



TEXT-FIG. 14. Fibroblast half an hour after treatment with Locke-Lewis solution (0.45 per cent sodium chloride content), showing one end rolled over onto the cell as if from contraction.

TEXT-FIG. 15. Normal fibroblast from a 7 day chick embryo heart; 4 days growth.

TEXT-FIGS. 16 to 21. The same cell after treatment with Locke-Lewis solution (1.8 per cent sodium chloride content). Text-fig. 16 shows long fine processes and a condensation of the cytoplasm. In Text-figs. 17 and 18 the processes have been withdrawn and blebs are forming. The mitochondria are shorter and more condensed. In Text-fig. 19, a large bleb surrounds the nucleus and granular cytoplasm. Text-figs. 20 and 21 show death processes. Vacuoles appear and the cell wall breaks down leaving only the nucleus and granular cytoplasm.

Locke-Lewis 0.45 per cent sodium chloride solution. Some were killed while others grew.

The effects following the addition of the hypotonic 0.45 per cent sodium chloride solution were varied. On some slides all the cells were killed; on others only a few, and migration went on beyond them; on still others the solution acted as a stimulant and growth and migration were greatly accelerated. Here again the condition of the cells in the original growth must have had a great influence on the results. While I tried to take only slides which had good reticular growths, there must have been great individual differences, as even in normal Locke-Lewis solution some cultures will live much longer than others made from the same heart, in the same medium, and at the same time.

Summary of Results with Hypotonic Solutions.

1. Locke-Lewis solutions were made hypotonic by the addition of distilled water. They were used with a sodium chloride content of 0.54, 0.45, 0.3, and 0.225 per cent respectively.

2. Tissue grew in the first two solutions, which acted as a stimulus.

3. The tissues did not live so long in these media as in normal Locke-Lewis solution, but growth was more rapid.

4. The cells of normal growth were killed by treatment with hypotonic solutions with a sodium chloride content of 0.3 and 0.225 per cent respectively.

5. The cells absorbed much water, as did also the nucleus, which frequently formed a nuclear vacuole as an outlet for the extra amount of liquid absorbed.

6. Neutral red vacuoles and granules soon lost their color when the cells were treated with the hypotonic solutions that caused their death.

7. Mitochondria were not affected by the hypotonic solutions, but as the cell died vesicles formed at the extremities and persisted after the rest of the mitochondrium had disappeared; or the mitochondria broke up into granules or simply became more slender until only a faint, rough outline was visible.

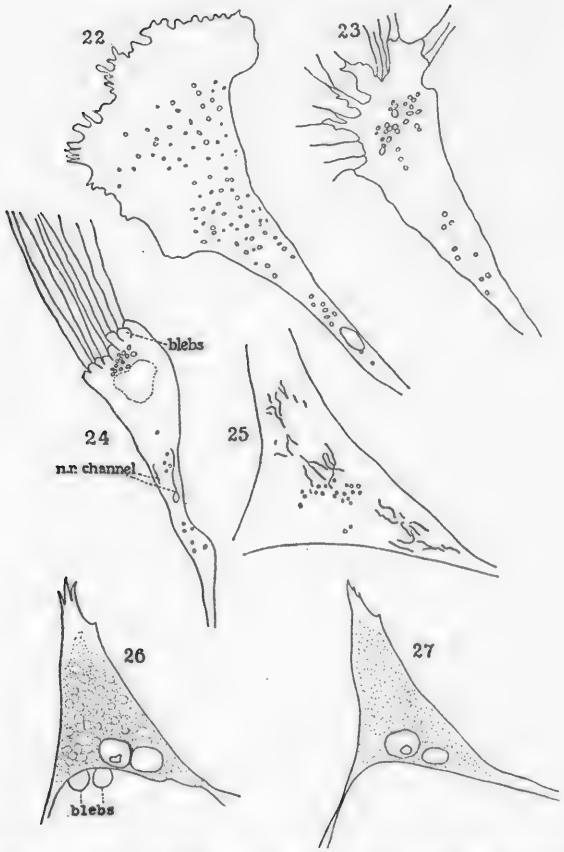
Experiments with Hypertonic Solutions.

Three grades of hypertonic Locke-Lewis solution, containing respectively 1.8, 1.5, and 1.2 per cent of sodium chloride, were used in this series of experiments.

Hypertonic Solution, 1.8 Per Cent Sodium Chloride.—Thirty-eight transplants were made, not one of which showed any signs of growth. It was evident that the fluid was too hypertonic to induce cell migration. Next the effect of this medium was tried upon tissue that had been growing well in normal Locke-Lewis solution for 2, 3, and 4 days respectively. In all the cells were killed within 3 or 4 hours, and in some within 1 hour after treatment.

The effect was followed on certain individual cells which were first stained with neutral red and Janus black No. 2. Text-fig. 15 is a cell taken from a 4 day growth of a 7 day chick heart after it had been treated with neutral red and Janus black at 11.15 a.m. At 11.20 the Locke-Lewis 1.8 per cent sodium chloride solution was added. By 11.25 the cell had begun to contract and crumple up like folds in a piece of cloth. At 11.30 (Text-fig. 16) the body of the cell was thick, and from one side and from the ends, where there had been processes, fine, thread-like structures extended out into the medium. By 11.40 (Text-fig. 17) most of these processes had been withdrawn; by 12.02 p.m. (Text-fig. 18) all the side processes had been withdrawn and the central granular part was surrounded by a clear, flat area. In Text-figs. 16 and 17 both mitochondria and neutral red granules are arranged as in normal growth. In Text-fig. 18 the mitochondria have become shorter and more concentrated in the center of the cell. At 12.15 (Text-fig. 19) the neutral red granules and mitochondria had disappeared and the cell was dead. Text-figs. 20 and 21, drawn at 12.20 and 12.40, show further death changes. In Text-fig. 20 large vacuoles are appearing in the clear areas. These later break down, leaving only the granular cytoplasm and nucleus (Text-fig. 21).

Text-figs. 22 to 24 show the contraction of the cytoplasm and the concentration of the neutral red granules at the end where the nucleus is located. The fine processes at the large end were invisible at 10.07 a.m. when the cell was treated with Locke-Lewis 1.8 per cent



TEXT-FIGS. 22 to 24. Cell from a 4 day growth of a 7 day chick embryo heart, stained with neutral red and treated with Locke-Lewis solution (1.8 per cent sodium chloride content). The processes at the anterior end have become visible in Text-fig. 22. Text-fig. 23 shows a contraction of the cytoplasm and a partial withdrawal of the anterior end to form thread-like processes. These processes are much longer in Text-fig. 24 where neutral red channels are shown opening into neutral red vacuoles.

TEXT-FIG. 25. Fibroblast from a 7 day chick heart; 4 days growth.

TEXT-FIGS. 26 and 27. The same cell after treatment with Locke-Lewis solution (1.8 per cent sodium chloride content). The alveolar structure of protoplasm is shown (Text-fig. 26), which 1 hour later (Text-fig. 27) became finely granular.

sodium chloride solution. By 10.10 (Text-fig. 22) they had contracted until they were plainly visible. Neutral red vacuoles are scattered over the cell, a very large one being in the posterior long process, only part of which was drawn. By 11.45 (Text-fig. 23) the cell had shrunken appreciably, the neutral red granules were concentrated at the anterior end, and thin processes were forming. At 12.35 p.m. (Text-fig. 24) these looked like long streamers. It appeared as though the anterior end of the cell had been attached at places to the cover-glass, and that when the cell began to shrink from loss of water the cytoplasm was pulled out into these long processes. When the pull was too great they lost hold and were rapidly withdrawn into the cell.

Coincident with the formation of these protoplasmic streamers, blebs—clear, round cytoplasmic protrusions—appeared behind them. Toward the center of the cell neutral red channels opened into the large neutral red vacuole. In many of the cells these channels were branching and anastomosing. This particular cell was dead at 1.45.

To those who still believe in the alveolar and reticular structure of protoplasm Text-figs. 25 and 26 will prove of interest. Text-fig. 25 shows a cell stained with Janus black and neutral red which bring out the mitochondria and the neutral red granules. At 11.20 a.m. the tissue was treated with Locke-Lewis 1.8 per cent sodium chloride solution. This cell did not contract so much as many of the others, nor were there formed the long, fine processes which I have found so characteristic of death in these hypertonic solutions. The cytoplasm, however, became alveolar (Text-fig. 26), small, round spaces appearing over the granular part of the cell by 12.25 p.m. The nuclear wall was very distinct, the granules no longer showed the neutral red color, and the mitochondria had disappeared—all evidences of cell death. By 1.45 (Text-fig. 27) these alveoli had also disappeared and the protoplasm had become granular. It would seem quite certain, therefore, that the alveolar structure described by the early cytologists was an artifact caused by the fixing solution, which was hypertonic to the protoplasm.

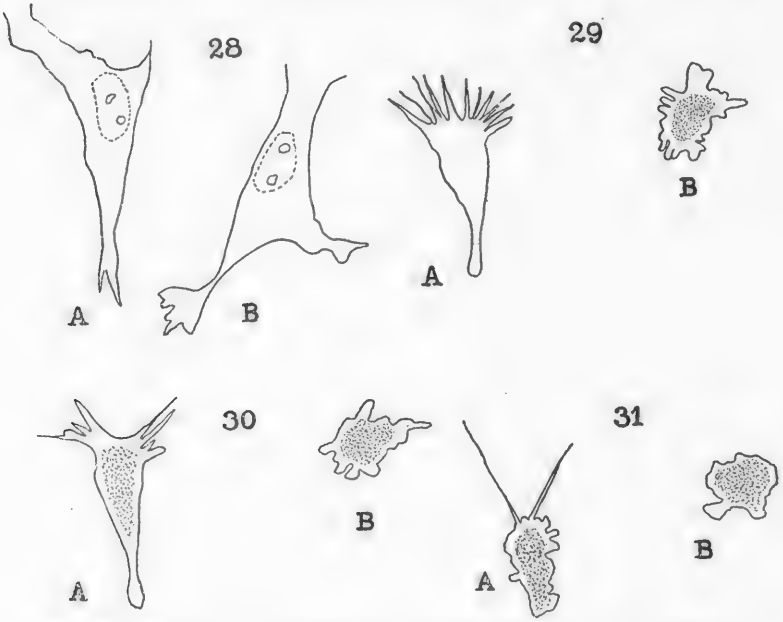
Hypertonic Solution, 1.5 Per Cent Sodium Chloride.—In spite of the fact that several authors have stated that tissues will not grow at all, or, at best, very poorly in hypertonic solutions, I have neverthe-

less grown a few in hypertonic Locke-Lewis solution with a sodium chloride content of 1.5 per cent. Out of 130 explants of heart tissue in this medium, twelve (9 per cent) showed migration and reticular formation. One of these lived 7 days. The migration was not great, and only in a few instances were there reticular growths.

I was interested in learning how the mitochondria would be affected by this new medium stained with Janus black No. 2 and neutral red. The mitochondria in many of the slides were long, irregular, and spread out normally into the processes of the cells. The neutral red granules were also arranged around the centriole and the mitochondria mingled with them as in normal cells. Occasionally the mitochondria were round or slightly oblong, but this was undoubtedly due more to the generally poor condition of the growth than to the hypertonic medium. The same round mitochondria are found on slides of Locke-Lewis solution when the growth is poor, and are probably due to some metabolic disturbance.

In order to observe the direct effect of this solution upon the normal cells I treated heart tissue, which was growing well in normal Locke-Lewis solution, with Locke-Lewis 1.5 per cent sodium chloride. In most cases it killed the cells. In some instances normal growths were used, other pieces of which were growing in the hypertonic 1.5 per cent solution, but the shock of this hypertonic solution to the delicate cells, thinly spread out on the cover-slip, was usually so great that they died. I noted especially the fate of two interesting cells (Text-fig. 28) in a 48 hour growth from a 10 day chick heart, which was treated with Locke-Lewis sodium chloride 1.5 per cent solution at 11.15 a.m. The reaction was immediate (Text-fig. 29). Their clear processes were retracted and formed long, fine processes on cell *A*, cell *B* contracting into an irregular mass. The fine processes on *A* were gradually withdrawn until all had disappeared (Text-figs. 30 to 33). *B* showed two distinct kinds of protoplasm—a clear, outer part, resembling the ectoplasm of an ameba and similarly active; and a denser, granular part in the center of the cell. This central mass was more stationary and the clear protoplasm flowed around it, either evenly, or forming blunt processes, which would be withdrawn and then form anew at another place, as described by Cash for cells treated with ether vapor. Later cell *A*

developed the same kind of movement (Text-fig. 34), but at noon was an inactive mass of cytoplasm. By 2.40 p.m. *B* had recovered completely; *A* was still an irregular mass, though the outer cytoplasm was again active (Text-fig. 35). By 4 p.m. (Text-fig. 36) both were active—*B* normal, *A* abnormal. The following day the cells had migrated and changed positions, so that it was impossible to say

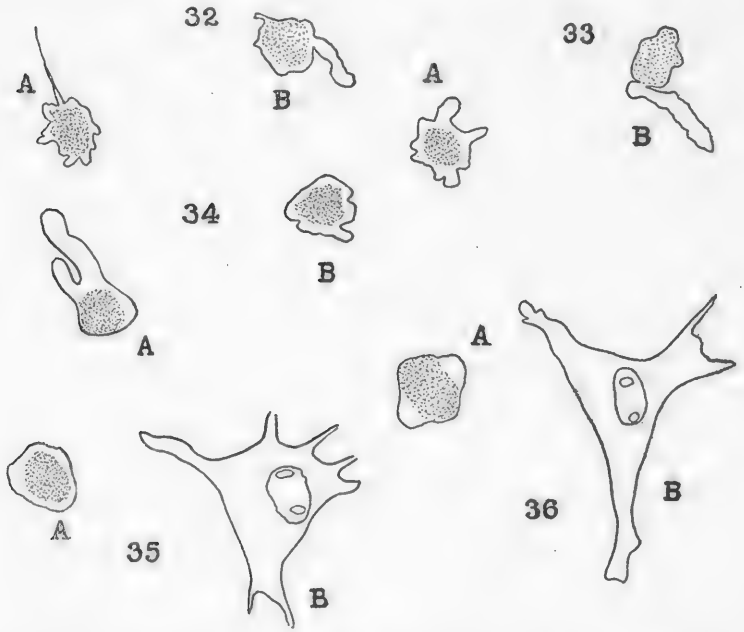


TEXT-FIG. 28. Two normal fibroblasts, *A* and *B*, before treatment with Locke-Lewis solution (1.5 per cent sodium chloride content) at 11.15 a.m.

TEXT-FIGS. 29 to 31. The same cells after treatment. In *A* is shown the formation of long fine processes, which were gradually withdrawn. *B* is an irregular mass showing two kinds of protoplasm, granular and clear, the latter moving like the pseudopodia of *Amœba proteus*.

whether these particular cells had entirely recovered. Half the number of cells on the slide were normal, with stellate forms; hence some of them had been able to withstand the shock. In another case which was followed one cell was apparently killed at once, while a larger

cell with a long process remained active, going through the stage of fine process formation and finally contracting, though the long posterior process remained extended like the posterior end of a *Vorticella*, acting as an anchor.



TEXT-FIGS. 32 to 36. Later stages of the cells shown in Text-figs. 28 to 31. The fine processes of *A* were completely withdrawn and after passing through an ameboid stage it became an irregular, rather inactive mass of protoplasm. *B* after the ameboid stage became normal.

Hypertonic Solution, 1.2 Per Cent Sodium Chloride.—Growth in this solution was very good; 32 per cent of the transplants grew, and one lived 15 days without being opened or treated in any way. Growth, however, was much slower than in the controls or in the hypotonic solutions. Often the cells did not begin to migrate until the 2nd or 3rd day, and then slowly, though in growths in which they were going to form a reticulum, they had done so by the 4th

day. Migration was not greater than in the hypotonic solutions but was often abundant, and the appearance of both migratory and reticular formations was that of healthy, normal cells.

When these cells were stained with neutral red and Janus black No. 2 the neutral red granules were found to be present in varying amounts and were arranged around the centriole, as in the controls. Usually there were some long mitochondria, but these varied in size.

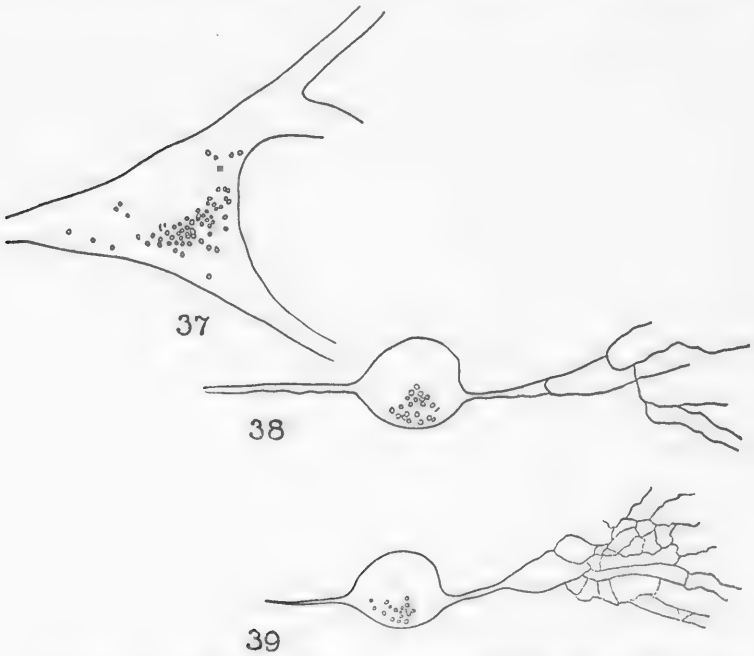
Occasionally the number of migrating cells was very small and these sometimes showed abnormalities. On one slide the cells extended out from the explant like a string of beads, three or four to a string. On another slide, which was later fixed in Zenker's fluid and stained with Ehrlich's hematoxylin, most of the cells were not flat but slightly rotund. The mitochondria were all round, a few neutral red granules were present, and the nucleus in many instances was apparently budding or dividing amitotically, in a manner similar to that shown in some of Macklin's figures. Some cells showed as many as three nuclei of different sizes, though all contained chromatin material. The metabolism of this culture had been seriously deranged, resulting in this change in the nuclear and mitochrondrial conditions.

The results following the addition of Locke-Lewis 1.2 per cent sodium chloride solution to normal growths of 1, 2, and 3 days were varied. The reaction was much slower than with the other hypertonic solutions. On three slides of 3 days growth few of the cells showed any contraction after 6 hours of treatment. Many were abnormal and contained a great number of deeply staining neutral red vacuoles. These cells lived and the vacuoles retained the neutral red stain for 1 to 3 days after treatment, or until some of the cells were 8 days old.

On other slides of 2 and 3 days growth interesting changes in the cells were observed. The long, fine, straight processes were also present here. Text-fig. 37 shows a normal cell which at 10.45 a.m. was treated with Locke-Lewis 1.2 per cent sodium chloride solution. At the end of half an hour little change was noted. Within the next hour the cell contracted and a fine network of protoplasmic processes formed at one side, quite a distance from the cell, yet connected with it by a long strand of clear protoplasm (Text-fig. 38).

By 2.10 p.m. (Text-fig. 39) this network had become more intricate, the neutral red granules had almost disappeared, and the cell was practically dead. Later, large clear vacuoles formed in the central mass.

In another cell which was followed the central mass contracted, leaving the clear cytoplasm in a large, thin sheet, stretching out at



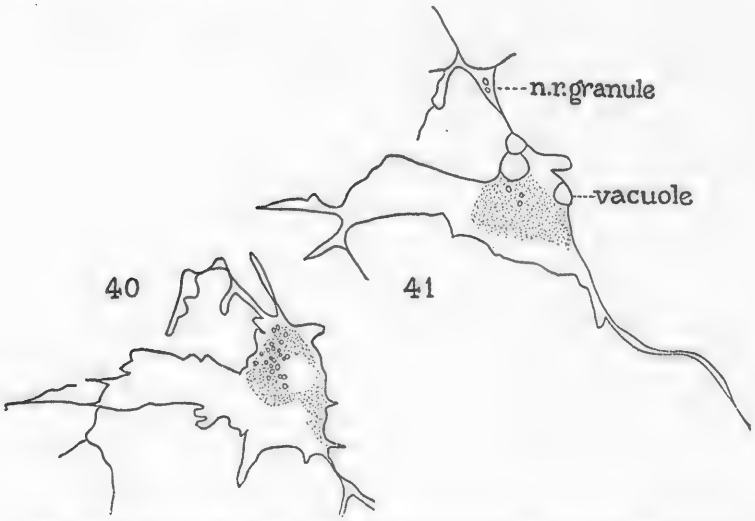
TEXT-FIG. 37. Normal fibroblast of a 7 day chick embryo heart; 3 days growth.

TEXT-FIGS. 38 and 39. The same cell after treatment with Locke-Lewis solution (1.2 per cent sodium chloride content), showing fine protoplasmic network developed from the processes of the cell.

one side of the cell with many slender processes extending in various directions. In Text-fig. 40 can be seen the concentration of neutral red granules in one part of the cell, while in Text-fig. 41 two of the granules have been caught in one of the processes when the central mass began to contract. Here blebs are forming and the large

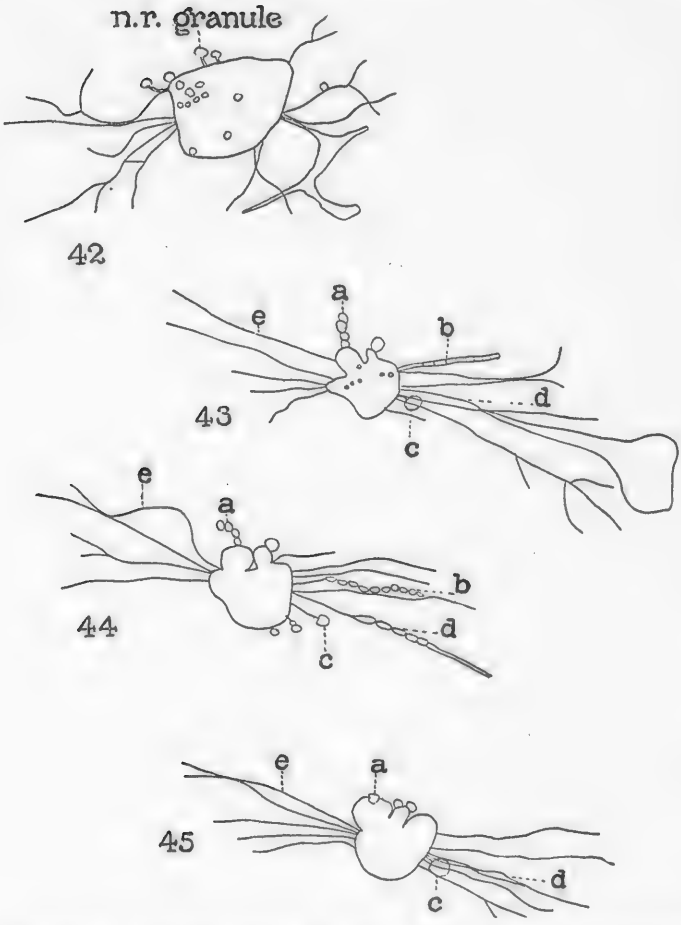
vacuoles indicate that the cell is nearly dead. In Text-fig. 42, from another cell, small neutral red granules are shown alongside of the processes and evidently held to them by fine protoplasmic threads, for they remain stationary though vibrating all the time with Brownian movement.

Still other interesting formations are seen in Text-figs. 43 to 45, from a 2 day growth of a 7 day chick heart. This cell was one of a



TEXT-FIGS. 40 and 41. Fibroblast after treatment with Locke-Lewis solution (1.2 per cent sodium chloride content), showing a contracted clear flat area of cytoplasm where normally there were processes. In Text-fig. 41 two neutral red granules have been caught in one of the processes during contraction of the cytoplasm.

group of three which had been followed after its treatment with Locke-Lewis 1.2 per cent sodium chloride solution at 11 a.m. In Text-fig. 43 at *a* there is a chain of protoplasmic beads which waved back and forth (Text-fig. 44) in the medium. Later, three of these beads broke away and disappeared. At *b* (Text-fig. 43) a slightly thicker process is seen which at 2.40 p.m. showed light and dark areas like a striated muscle. These quickly became constricted into a chain of proto-



TEXT-FIG. 42. Fibroblast treated like that in Text-fig. 40, showing neutral red granules fastened to the cell by slender threads of cytoplasm.

TEXT-FIGS. 43 to 45. Fibroblast treated with Locke-Lewis solution (1.2 per cent sodium chloride content). Many of the fine processes showed movement. The beaded formations at *a* and *b* moved back and forth; *c* was continually oscillating; *d* was moving and *e* waved until it became attached to another fibril (Text-fig. 44); then it moved like an undulating membrane until the cell was dead.

plasmic beads which were free moving and waved about in the surrounding medium (Text-fig. 44). At 3.00 the process broke loose, became entangled with the other thin processes, and disappeared. *c* was a round process of protoplasm attached by a fine thread, which also oscillated back and forth. *d* was apparently attached to a process from a neighboring cell, and in Text-fig. 43 was only a thick process. In Text-fig. 44 it had become beaded, the beads being of different sizes, and was undulating gently. Later (Text-fig. 45) it had become practically straight again, and as the cell was dying the movement gradually lessened. *e* always remained a thin, thread-like process. At first free moving (Text-fig. 43), it later became attached to another process (Text-fig. 44) which was stationary. However, it continued to wave like an undulating membrane until nearly 4 p.m., when motion in the processes ceased and the cell was dead, the color in the neutral red granules having also disappeared.

This long, waving fiber answers the description of those grown by Baitsell in plasma. He believes that they are not outgrowths of the embedded tissue but come from fibrin in the plasma clot. His evidence for this is not complete, as his stains give two different results, one confirmatory of his theory, the other pointing toward the cellular origin of the fibers. In the present experiments there was no plasma and consequently no fibrin. Furthermore, this fibril is directly connected with the cell and remains in active motion only as long as the cell is alive. Its place of origin, the clear, protoplasmic area which contracts to form long, fine processes, is the same as that given by Lewis (1917). She describes the formation of the fibrils of connective tissue from the ectoplasm of the cell.

Summary of Results with Hypertonic Solutions.

1. Hypertonic solutions were made by boiling down Locke-Lewis solution until the sodium chloride content was 1.2, 1.5, and 1.8 per cent respectively.

2. Tissues grew in the first two of these solutions.

3. Tissues did not live so long in these solutions as in normal Locke-Lewis solution and growth was slower.

4. The cells of normal growth were killed by treatment with hypertonic solutions with a sodium chloride content of 1.8 and 1.5 per cent.

5. When treated with hypertonic solutions the cells usually contracted, their thin processes became long and thread-like and were later drawn into the body of the cell.

6. Connective tissue fibrils formed from these thread-like processes. They moved and anastomosed with other fibrils.

7. Neutral red channels formed in many cells.

8. The cytoplasm frequently became alveolar when the death process set in.

9. These three hypertonic solutions showed a definite gradation in their effects on the processes of the fibroblast. In Locke-Lewis solution containing 1.8 per cent sodium chloride the processes contracted rapidly, leaving many thread-like structures in their places. These were quickly withdrawn and the cell soon died. In Locke-Lewis solution containing 1.5 per cent sodium chloride the thread-like processes were frequently formed, but the cells did not all die; some recovered. In Locke-Lewis solution containing 1.2 per cent sodium chloride the processes still formed but more slowly. They also showed motion, which lasted as long as the cell was alive.

Hypertonic versus Hypotonic Solutions.

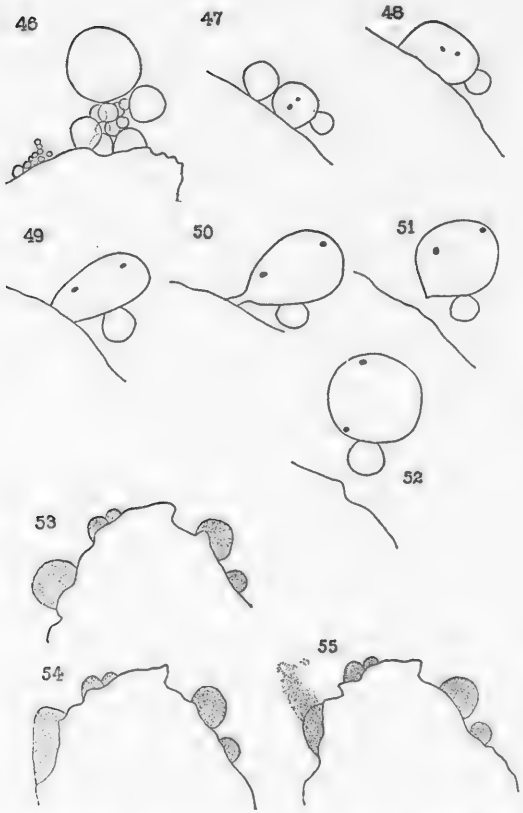
In comparing the growth in hypertonic with that in hypotonic media the following points may be emphasized. In the former the migration is slower than in the latter. In hypertonic solutions the optimum growth is reached on the 3rd day or later, in hypotonic solutions on the 2nd day. The growths live longer in hypertonic solutions but are, as a rule, smaller than in hypotonic solutions. Table I shows this difference of growth in the various media.

TABLE I.

Amount of NaCl in medium.	Total No. of explants.	No. that grew.	No. that died.	Growth.	Greatest No. of days any culture lived in the medium.
per cent				*per cent*	
1.8	38	0	38	0	0
1.5	130	12	118	9	7
1.2	100	32	68	32	15
0.9	282	188	94	66.6	19
0.54	23	20	3	86.9	7
0.45	202	90	106	44.5	12
0.225	12	0	12	0	0

There seemed to be a possibility of proving whether or not the neutral red granules and vacuoles are the product of metabolism, as Lewis (1919) believes, by an experiment in which parts of the same heart were grown respectively in Locke-Lewis normal solution, Locke-Lewis solution with sodium chloride content of 0.45 per cent, and with 1.2 per cent sodium chloride content, the rate of growth being different in each of these media. Accordingly, pieces of heart from a 7 day chick embryo were planted in the three solutions and incubated for 48 hours. At the end of 24 hours nine of the normal Locke-Lewis cultures, eight of the 0.45 per cent sodium chloride, and two of the 1.2 per cent sodium chloride showed growth. After another 24 hours of incubation eight of the Locke-Lewis, four of the 0.45 per cent sodium chloride, and five of the 1.2 per cent sodium chloride cultures were alive. Here is seen again the slow growth in hypertonic solutions. These cultures were stained with neutral red and Janus black No. 2. Growth in both the Locke-Lewis solution and 1.2 per cent sodium chloride was good, and each showed a normal arrangement of the neutral red granules. In the hypotonic solution growth had been poor. Few cells had migrated, and in these the large neutral red vacuoles were full of deeply stained material. The cells, in their poor condition, were evidently having difficulty in getting rid of the waste products which were accumulating from metabolism.

In working with hypertonic solutions, occasionally with normal Locke-Lewis solution, and in a few instances with hypotonic solutions, I observed an interesting attempt on the part of the tissue to adapt itself to the new medium. Tissues were planted in Locke-Lewis solution containing 1.8 and 1.5 per cent sodium chloride. On the following day transparent, balloon-like structures were noted, varying in size from quite small to very large, which formed along the edges of the culture. Some explants had many of these balloons around the sides, others only a few large ones. Sometimes balloons of all sizes piled up in masses (Text-fig. 46), those at the top being free from the tissue. Upon close observation these structures were seen to begin as small hemispheres rising out of the explant. In time they became almost spherical and increased in size as though something from the tissue was being poured into them. Occa-



TEXT-FIG. 46. Masses of balloons of all sizes piled up at the edge of the heart tissue which had been planted in Locke-Lewis solution of 1.5 per cent sodium chloride content 48 hours before.

TEXT-FIGS. 47 to 52. The formation of a large balloon by the fusion of two small ones (Text-figs. 47 and 48). This large one elongated (Text-fig. 49), formed a stalk (Text-fig. 50), was pinched off (Text-fig. 51), and became free floating (Text-fig. 52).

TEXT-FIG. 53. Granular hills along the edge of a piece of heart planted in Locke-Lewis solution of 1.5 per cent sodium chloride content.

TEXT-FIGS. 54 and 55. One of these granular hills enlarged and discharged its contents into the surrounding medium.

sionally two fused together, as shown in Text-figs. 47 and 48. This particular one continued to increase in size until the pressure became so great that it elongated (Text-fig. 49). A small stalk was formed (Text-fig. 50) which quickly snapped, thus freeing the balloon (Text-fig. 51). It rounded up into a ball within a few seconds (Text-fig. 52) and rolled around over the tissue when the slide was tilted back and forth. It appeared to be taking in culture medium or else flattening out, as it continued to increase in size until one side grew faint, then disappeared, and the balloon went to pieces.

Some cultures were full of these balloons floating around singly or in masses. They were frequently found being given off on the 2nd day, but were not seen to form after that, though those already formed persisted for several days. One slide 11 days old still showed masses of them, but these disappeared on the 12th day. The balloons were clear and transparent, and occasionally contained small particles like cells. There was a distinct surface tension which quickly rounded them up after they were given off. They were also very delicate structures, as evidenced by the fact that when attempts were made to transfer the cover-slip, with the culture in its hanging drop, from the depression slide with the vaseline ring to a straight slide with a large drop of the same hypertonic solution in which the tissue was planted, the balloons nearly always went to pieces, no matter how carefully the cover-slip was lowered onto the new drop of medium. Occasionally this transfer was successful, and on placing the slide under the microscope with dark-field illumination the balloons showed as small, milky white structures not only at the edges, as had appeared with the ordinary illumination, but all over the explant.

There was another type of structure, which I have termed granular hills, that also appeared over the explants. These were more stable and lasted as long as the cultures were kept. They grew in size, sometimes becoming quite large, though they were most frequently seen as small balls or hills along the edges or between the angles of the tissue (Text-fig. 53). They were very finely granular. Sometimes the surface tension would be taxed too much and the granular hill would break open at one place, pouring the fine granules into the surrounding medium (Text-figs. 53 to 55).

Both balloons and granular hills would appear on the same explant. These explants never showed growth or migration of cells, though they would often continue to beat for several days. The structures described above were evidently a means of adjusting the tissue to its new medium. Some days every tissue planted in Locke-Lewis 1.8 and 1.5 per cent sodium chloride solutions would show the balloons or the granular hills, sometimes both. Again, a new medium, made in the same proportions of Locke solution, bouillon, and dextrose, boiled down to the same volume as that used in the preceding experiments, would give few if any of these structures, and also practically no growth. The balloons and granular hills were not the large precipitations, with irregular, angular outlines, which are frequently observed in tissue cultures and which are much more coarsely granular. They may be the same material which Burrows and Neymann state is liberated from the cells when they are removed from their normal habitat to an oxygen-containing plasma or salt solution. These authors state that the substances are "almost transparent, their refraction not very different from that of the original medium, and they accumulate at the surface of the medium to form a membrane." Perhaps the growth in plasma with a different osmotic pressure from Locke-Lewis solution may account for the membrane formation instead of the balloons and granular hills found in my experiments. On the other hand, they may be quite different substances, as Burrows and Neymann state that theirs "are liberated in large amounts from a tissue fragment rich in cells." While the structures described above rarely appeared when there was cell migration, a few times I have found near the explant, after the cells had degenerated, granular hills which had been completely covered over by the cells.

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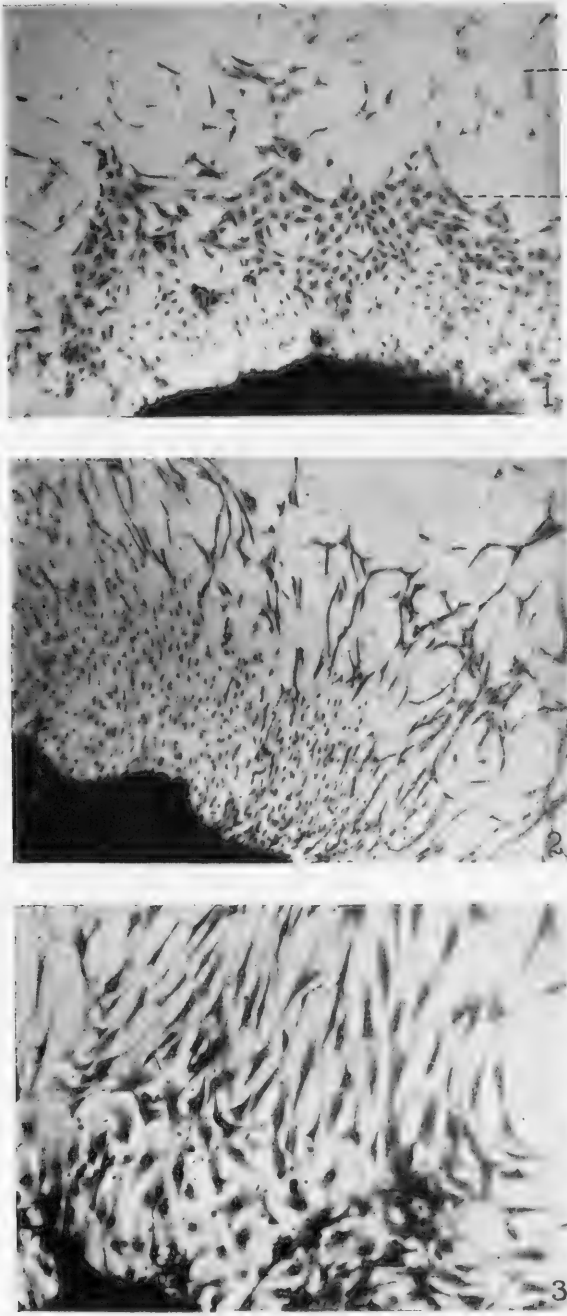
EXPLANATION OF PLATES.

PLATE 56.

FIG. 1. Photomicrograph of normal growth of chick heart tissue, showing fibroblasts at *a* in migration formation, and mesothelial cell in membrane formation at *b*.

FIG. 2. Photomicrograph of 3 day growth of chick heart tissue in hypotonic Locke-Lewis solution (0.45 per cent sodium chloride), showing the early death of cells next to the explant.

FIG. 3. Photomicrograph of a normal 48 hour growth which was treated with hypotonic Locke-Lewis solution (0.45 per cent sodium chloride) and incubated for 24 hours. Some of the cells next to the explant have been killed, but others are migrating out over them.



(Hogue: Fibroblasts of embryo chick hearts.)

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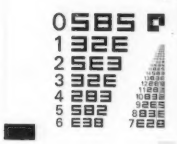
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